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Effect of porcine circovirus type 2 on porcine cell populations

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Effect of porcine circovirus type 2 on porcine cell populations

by

Shan Yu

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Veterinary Microbiology

Program of Study Committee:
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Michael Wannemuehler
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Iowa State University
Ames, Iowa
2006
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Major Professor

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For the Major Program
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LIST OF ABBREVIATIONS

7AAD 7-amino-actinomycin D
AI  apoptotic index
AN  Annexin V
ANOVA Analysis of variance
BAL bronchoalveolar lavage
BFDV beak and feather disease virus
BHQ_1 Black Hole Quencher 1
BLN bronchial lymph nodes
BSA bovine serum albumin
CaCV canary circovirus
Cap capsid protein
Cap mRNA spliced capsid mRNA
CAV chicken anemia virus
CCasp3 cleaved caspase-3
CD25 IL-2 R alpha
CFSE 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester
CoCV/PiCV pigeon or columbid circovirus
ConA concanavalin A
CPE cytopathic effect
CT congenital tremors
CT threshold cycle
DCs dendritic cells
DEX dexamethasone
DPI days post-infection
DuCV duck circovirus
ELISA enzyme-linked immunosorbent assay
FAM 6-carboxyfluorescein
FBS fetal bovine serum
FCM fetal cardiomyocytes
FITC fluorescein isothiocyanate
GoCV goose circovirus
HIV human immunodeficiency virus
HPI hours post-infection
IFA immunofluorescence assay
IFN interferon
IHC immunohistochemistry
IL interleukin
ILN superficial inguinal lymph nodes
IPMA immunoperoxidase monolayer assay
ISH in situ hybridization
<table>
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<tr>
<th>Abbreviation</th>
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<tr>
<td>KLH</td>
<td>keyhole limpet hemocyanin</td>
</tr>
<tr>
<td>MCP-1</td>
<td>monocyte chemoattractant protein-1</td>
</tr>
<tr>
<td>MIP-1</td>
<td>macrophage inflammatory protein-1</td>
</tr>
<tr>
<td>MOI</td>
<td>multiplicity of infection</td>
</tr>
<tr>
<td>NS</td>
<td>non-structure</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
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<tr>
<td>ORF2</td>
<td>open reading frame 2</td>
</tr>
<tr>
<td>ORFs</td>
<td>open-reading frames</td>
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<tr>
<td>PAMs</td>
<td>pulmonary alveolar macrophages</td>
</tr>
<tr>
<td>PBMCs</td>
<td>peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
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<tr>
<td>PBS-T</td>
<td>Tween 20 in PBS</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<td>PCV</td>
<td>procine circovirus</td>
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<td>PCV type 1</td>
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<td>PCV2</td>
<td>PCV type 2</td>
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<tr>
<td>PDNS</td>
<td>porcine dermatitis and nephropathy syndrome</td>
</tr>
<tr>
<td>PHA</td>
<td>phytohemagglutinin</td>
</tr>
<tr>
<td>PI</td>
<td>propidium iodide</td>
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<tr>
<td>PK-15</td>
<td>porcine kidney cell line</td>
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<td>PMWS</td>
<td>postweaning multisystemic wasting syndrome</td>
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<td>PPV</td>
<td>porcine parvovirus</td>
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<td>PRDC</td>
<td>porcine respiratory disease complex</td>
</tr>
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<td>PRRSV</td>
<td>porcine reproductive and respiratory syndrome virus</td>
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<td>PWM</td>
<td>pokeweed mitogen</td>
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<tr>
<td>Rep</td>
<td>replication-associated protein</td>
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<td>RF</td>
<td>replication form</td>
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<td>coefficients of correlation</td>
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<td>reverse transcriptase</td>
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<td>RT-PCR</td>
<td>reverse transcription polymerase chain reaction</td>
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<td>SEW</td>
<td>segregated early weaned</td>
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<tr>
<td>SIV</td>
<td>swine influenza virus</td>
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<tr>
<td>S/P ratio</td>
<td>sample-to-positive ratio</td>
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<tr>
<td>ss</td>
<td>single-stranded</td>
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<tr>
<td>TCID$_{50}$</td>
<td>50% tissue culture infective dose</td>
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<tr>
<td>TdT</td>
<td>terminal deoxynucleotidyl transferase</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>TTV</td>
<td>TT virus</td>
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<tr>
<td>TUNEL</td>
<td>terminal deoxynucleotidyl transferase-mediated dUTP-nick end labeling</td>
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CHAPTER 1. GENERAL INTRODUCTION

Introduction

The porcine circovirus (PCV) is a small, non-enveloped, single-stranded (ss), closed circular DNA virus. Two genotypes of PCV have been identified. PCV type 1 (PCV1) was first detected as a contaminant of the porcine kidney cell line (PK-15) (CCL-33) (Tischer et al., 1974). In 1991, a new disease named postweaning multisystemic wasting syndrome (PMWS) was identified in a swine herd in Canada (Clark, 1997; Harding, 1996). The cause of this disease was attributed to a new PCV variant, PCV type 2 (PCV2). Since 1991, PMWS has been reported in many countries (Allan and Ellis, 2000). The overall DNA sequence identity within PCV1 or PCV2 isolates is greater than 90%, while the identity between PCV1 and PCV2 isolates is 68–76% (Fenaux et al., 2000; Hamel et al., 1998). The genome organization of PCV1 and PCV2 consists of two major open-reading frames (ORFs), ORF1 and ORF2. ORF1 encodes the replication-associated protein (Rep) and ORF2 encodes the major capsid protein (Cap) (Liu et al., 2001; Nawagitgul et al., 2000). The Rep genes of PCV1 and PCV2 are highly conserved, with 83% identity at the nucleotide sequence level, whereas the Cap genes share only 67% identity (Mankertz et al., 1998a).

Depletion of lymphocytes in the lymphoid follicles and their replacement by macrophages in conjunction with the presence of PCV2 viral antigen is a consistent finding associated with PMWS (Allan et al., 1999b; Meehan et al., 1998). Alteration in the populations of the macrophages and lymphocytes in the lymphoid tissues of infected pigs indicates that the virus may infect and has an impact on these immune cells. Although PCV2 has primarily been associated with PMWS, it has also been implicated in a number of disease syndromes including porcine dermatitis and nephropathy syndrome (PDNS), porcine
respiratory disease complex (PRDC), enteritis, reproductive failure, and congenital tremors (CT) type AII (Allan and Ellis, 2000; Segales et al., 2004b). Preventing and controlling these disease syndromes makes it important to understand the pathogenesis of PCV2 in infected pigs. The objectives in the studies described herein were threefold: 1) to evaluate PCV2 replication in populations of immune cells in vitro and in vivo; 2) investigate the effects of PCV2 replication on activated immune cells in vitro and; 3) investigate the relationship between PCV2 replication and cellular proliferation.

Several methods such as virus isolation, indirect immunofluorescence, in situ hybridization (ISH), immunohistochemistry (IHC) and polymerase chain reaction (PCR) have been used to detect PCV2 in tissues or serum samples (Allan et al., 1998b; Choi et al., 2000; Ellis et al., 1998; McNeilly et al., 1999; Quintana et al., 2001; Rosell et al., 1999; Tischer et al., 1987). However, these assays lack of sensitivity, time-consuming, or unable to discriminate between the PCV2 virons and replicating virus. Transcriptional analysis of PCV2 identified one capsid mRNA and a cluster of five Rep-associated RNAs, which increase in a time-dependent manner during PCV2 replication in PK-15 cells (Cheung, 2003c). The capsid mRNA contains a splice junction at nucleotides 361 and 1737 (Cheung, 2003c). In our first study, primers were designed to amplify across the capsid mRNA splice junction and a PCV2-specific reverse transcription polymerase chain reaction (RT-PCR) assay able to differentiate between PCV2 replication products and virus DNA in PK-15 cells was developed (Yu et al., 2005). Because this assay did not allow us to quantify the viral product, a quantitative real-time RT-PCR assay to detect the spliced capsid mRNA (Cap mRNA) was developed in the second study. It has been shown that the viremia caused by PCV2 is both cell-free and cell-associated, but the cell-associated form appears to be more
apparent and long-lasting (Pensaert et al., 2004). However, it is unknown whether the PCV2 associated with monocytes isolated from peripheral blood mononuclear cells (PBMCs) was the result of PCV2 active replication within the different cell populations or was due to non-replicating virus that had either adsorbed to or been endocytosed by the cells. Moreover, it has been reported that general stimulation of the immune system could increase PCV2 replication and predispose pigs to PMWS (Grasland et al., 2005; Krakowka et al., 2001; Kyriakis et al., 2002). Therefore, in the second study, we assessed PCV2 replication in cultured PBMCs with or without mitogen stimulation at different time points. To further identify the specific PBMC populations in which PCV2 replicates, stimulated and infected PBMCs were sorted by flow cytometry into CD3⁺ (T lymphocytes), SWC3⁺ (monocytes) and SWC3⁻ (PBMCs with monocytes removed) cell populations. The various populations were evaluated for PCV2 Cap mRNA and viral DNA to ascertain the capability of these cell populations to support PCV2 replication.

Even though PCV2 antigen and DNA have been detected from multiple organs and multiple cell types in infected pigs (Chae, 2004; Chianini et al., 2003; Choi and Chae, 1999; Hamel et al., 2000), it remains unclear which tissue or cell type is the primary site of virus infection and replication. Therefore, the aim of our third study was to understand the replication kinetics of PCV2 in vivo. In this study, the levels of PCV2 Cap mRNA and viral DNA was assessed in PBMCs, bronchoalveolar lavage (BAL) cells and tissue samples including bronchial lymph nodes (BLN), superficial inguinal lymph nodes (ILN), lung, tonsil, thymus, spleen, liver and kidney from pigs at early stage of PCV2 infection. Viral loads in serum/BAL cell-free fluid, microscopic lesions and PCV2 antigen in tested tissues, PCV2 antibody levels in serum/BAL samples were also investigated using real-time PCR,
histopathology, IHC and ELISA assays, respectively. In addition, PCV2 infection and replication in different immune cell populations isolated from peripheral blood or BLN mononuclear cells from infected pigs were separated by magnetic cell sorting (AutoMACS) and the Cap mRNA and viral DNA levels were determined. The findings from this study identified the cell populations in which PCV2 infects and replicates in pigs at early stage of PCV2 infection, thus, increasing the knowledge of the kinetics of PCV2 replication in infected pigs.

Lymphocyte depletion of follicular and interfollicular areas together with macrophage infiltration of lymphoid tissues is the hallmark lesion associated with PMWS in PCV2 infected pigs (Allan and Ellis, 2000). These findings are highly correlated with a decrease in circulating B and T lymphocytes and reduced numbers of these cells in lymphoid organs (Darwich et al., 2002; Segales et al., 2001). Moreover, previous studies have observed a strong correlation between the amount of PCV2 nucleic acid or antigen and the severity of lymphoid depletion and granulomatous changes in lymphoid tissues (Segales and Domingo, 2002). Though it has been suggested that PCV2 induces apoptosis that results in B lymphocyte depletion (Shibahara et al., 2000), recent research comparing cell populations in ILN from normal pigs and pigs with lesions consistent with PMWS found decreased cell proliferation in the absence of increased apoptosis suggesting that apoptosis was not an important mechanism leading to cell depletion (Mandrioli et al., 2004). However, this study was based on tissues diagnosed as PMWS under field conditions, which may caused by multiple pathogens not by PCV2 alone. In contrast to the theory of reduced proliferation in response to PCV2 infection, research has also suggested that vaccination and stimulation of an immune response may enhance the replication of PCV2 and predispose pigs to PMWS.
Lymphocytes proliferate when activated by antigens or adjuvants and results in an increase of PCV2 replication in them as they divide. Thus, conflicting theories are existent regarding the pathogenesis of the lymphoid depletion associated with PCV2 infection. To date, the mechanism responsible for lymphoid depletion remains unknown. Therefore, the objective of the fourth study was to evaluate the effects of PCV2 infection and replication on activated PBMCs in vitro as well as the relationship between PCV2 replication and cellular proliferation. Viral replication was assessed in infected PBMCs in the presence of the mitogens, concanavalin A (ConA) or pokeweed mitogen (PWM). ConA and PWM have been reported to stimulate different subpopulation of T lymphocytes. In addition, PWM was used to stimulate porcine B lymphocytes (Dorn et al., 2002; Williams, 1993). We also measured lymphocyte proliferation using 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (CFSE) labeling and determined the apoptotic index by Annexin V (AN) and 7-amino-actinomycin D (7AAD) staining using flow cytometric analysis. To further study whether the proliferation of PBMCs facilitate PCV2 replication, the levels of PCV2 Cap mRNA was assessed in PBMCs that had been stimulated with ConA for 12, 36, and 72 hours prior to inoculation of PCV2 for 24 hours. In addition, the level of PCV2 Cap mRNA in proliferated and non-proliferated PBMCs with ConA or PWM stimulation was also evaluated. Determination of the apoptotic and proliferation indices on PCV2 infection and replication in the immune cells should further increase our understanding of the pathogenesis of PCV2-associated diseases.

Dissertation Organization

This dissertation is comprised of six chapters in the alternative format. Chapter 1 is a general introduction containing the introduction, dissertation organization and literature
review. Chapter 2 describes the development of a reverse transcription-PCR assay to detect porcine circovirus type 2 transcription as a measure of replication. This chapter was published as a short communication in the Journal of Virological Methods. Chapters 3 through 5 are the author’s research, in manuscript format prepared for publication. The sixth Chapter presents the general conclusions of the entire research. The citations from the introduction, literature review, and general conclusion sections are compiled in the reference list at the end of this dissertation.

**Literature Review**

**PCV1 and PCV2**

**Background of PCV**

Porcine circovirus (PCV) was first detected as a contaminant of a continuous porcine kidney cell line (PK-15) (Tischer et al., 1974). This cell culture isolate was shown to possess a circular single-stranded DNA genome of 1.76 kb (Tischer et al., 1982; Buhk et al., 1985; Meehan et al., 1997). In 1997, the presence of PCV antigen was demonstrated in lesions of pigs with a clinical disorder called postweaning multisystemic wasting syndrome (PMWS) (Clark, 1997). Nucleotide sequence analysis of PCV associated with PMWS revealed important differences compared to the previously known PCV isolated from PK-15 cells (ATCC CCL-33) (Hamel et al., 1998), and it was decided that the viruses would be identified as PCV type 1 (PCV1) for the original PCV contaminant of PK-15 cells, and PCV type 2 (PCV2) for the virus associated with the new disease syndrome, PMWS (Allan et al., 1999b; Meehan et al., 1998).

On the basis of their morphology and circular ssDNA genome, PCV1 and PCV2 were classified in the *Circoviridae* family which comprises two genera *Circovirus* and *Gyrovirus*
The genus *Circovirus* include PCV1 and PCV2, beak and feather disease virus (BFDV), pigeon or columbid circovirus (CoCV/PiCV), duck circovirus (DuCV), goose circovirus (GoCV), and canary circovirus (CaCV) (Ritchie et al., 1989; Todd et al., 1991; Woods et al., 1993; Todd et al., 2001; Phenix et al., 2001). All members of this genus have the typical ambisense genomic structure of a circovirus. The genus *Gyrovirus* consists only of chicken anaemia virus (CAV) (Pringle, 1999). Studies showed that PCVs are closely related to plant circoviruses, now renamed nanoviruses (Meehan et al., 1997). It has been suggested that the PCV genome is an intermediate between plant geminivirus and nanovirus (Niagro et al., 1998) and that PCV may be the result of a recombination event between a nanovirus and an animal picorna-like RNA virus (Gibbs and Weiller, 1999).

**Biological and physicochemical properties**

It is known that PCV1 has a buoyant density of 1.37 g/cc in CsCl, is not able to haemagglutinate erythrocytes from a wide range of species, is resistant to inactivation at pH=3 and by chloroform, and is stable at temperatures of 56 °C to 70 °C for at least 15 min (Allan et al., 1994c). The biological and physicochemical characteristics of PCV2 are similar to those of PCV1. Both PCV1 and PCV2 are highly resistant to inactivation by common detergents and disinfectants (Allan et al., 1994b; Royer R.L., 2001).

PK-15 cells are the most commonly used cell line for propagating PCV. As might be expected, the PK-15 cell-derived PCV1 replicates more efficiently in PK-15 cells than does PCV2 (Fenaux et al., 2003). The replication kinetics of different PCV2 strains has been compared in PK-15 cells, and it was shown that the abortion-associated PCV2 strains have different replication kinetics than viruses isolated from cases of PMWS or porcine dermatitis and nephropathy syndrome (PDNS) (Meerts et al., 2005).
A cytopathic effect (CPE) induced by PCV infection has not been reported, and as a result, cell infection is normally monitored by immunofluorescent or immunoperoxidase staining of infected cell cultures (Allan et al., 1994a; Tischer et al., 1987). Investigation of PCV1 replication in porcine, bovine, ovine, avian or human cell cultures showed that all porcine derived cell cultures were susceptible to infection with PCV1 (Allan et al., 1994c). Primary, semi-continuous and continuous cultures derived from the other species tested in the study were found to be also susceptible to infection, with the exception of avian cell cultures. Moreover, viral antigen was detected in the cytoplasm of many susceptible cells while nuclear staining was detected in only a very few cells in porcine-derived cell or Vero cell cultures (Allan et al., 1994c). The results of infectivity titrations following passage of cell lysates indicate that production of infectious virus in inoculated cell cultures was confined to porcine-derived and Vero cultures (Allan et al., 1994c). In addition, the replication of PCV1 in porcine and bovine monocyte/macrophage culture has been reported (Allan et al., 1994b). PCV1 DNA replication has been shown to be dependent on cellular enzymes expressed during S phase of growth and in the natural multiplication cycle in vitro, PCV DNA replication begins only when cells have passed mitosis (Tischer et al., 1987).

Recently, a study investigated the susceptibility of human cells to PCV in vitro (Hattermann et al., 2004). Nine adherent human cell lines, Chang liver, FL, 293, Hep2, RH, CaCo, HeLa, Ma23, and Rd, were either transfected with religated DNA of PCV1 or PCV2 or infected with the viruses. After transfection, Chang liver, 293, Hep2, HeLa, and RH cells maintained PCV1 and PCV2 DNA and expressed the viral antigen as well. PCV2 DNA was lost after 2 weeks, while PCV1 persisted in all cells until the end of the 6-week experimental period. Expression rates of viral proteins were comparable with those observed in PK-15
cells after transfection of the PCV genome. A CPE was observed in the PCV2-transfected human cells, but not in PCV1-infected cells. In contrast to the results obtained by transfecting cells with PCV-DNA, infection with PCV1 was observed in Chang liver, 293, and Hep2 cells, while Rd cells were found to be susceptible to PCV2 (Hattermann et al., 2004). In vivo, PCV is often found in cells of the macrophage/monocyte lineage. Therefore, eight human suspension cell lines Wil2, THP1, Jurkat, Molt4, C8166, CEM, U937, H9 and human PBMCs with PCV1 and PCV2 infection were further evaluated. While viral antigen was observed in the positive control of the porcine lymphoblast cell line, L23, no PCV DNA or antigen was observed in tested cell lines, indicating that they are non-permissive for PCV replication (Hattermann et al., 2004). In addition, four animal cell lines (simian Vero cells, murine cell lines RAT-2 and CTL, and feline cell line PG4) were infected with PCV and investigated for expression of viral proteins in immunofluorescence assay (IFA). Fluorescence signal was observed in Vero cells, while the murine cell lines, RAT-2 and CTL, remained negative, as well as feline cell line, PG4. Subcellular localization of the Cap protein was observed by IFA and confocal microscopy in PCV-infected cells. The fluorescence was observed in PCV-permissive PK-15 cells with fluorescence in the nucleus, in the nucleus with nucleoli spared, or restricted to the cytoplasm. In contrast, PCV-infected human cells had Cap protein accumulated punctiformly in the nucleus, suggesting that protein transport or virus assembly may be perturbed in these cells (Hattermann et al., 2004). Overall, these studies of PCV infection and replication in different cell lines from different species demonstrated that PCV1 can be better propagated those cell lines than PCV2. However, most cell lines are not permissive for PCV.
Serological studies

Serum antibodies to PCV1 have been demonstrated in pigs in Germany (Tischer et al., 1995), Canada (Dulac and Afshar, 1989), Great Britain (Edwards and Sands, 1994), and Northern Ireland (Allan et al., 1994c) suggesting PCV1 infection of pigs is ubiquitous throughout the world. An absence of serum antibodies to PCV1 in cattle, sheep, chickens, turkeys, goats, mice, rabbits, or humans has also been reported (Allan et al., 1994b). However, in contrast, serum antibodies to PCV1 were detected in humans, mice and cattle in Germany, using a combination of indirect immunofluorescence and enzyme-linked immunosorbent assay (ELISA) procedures (Tischer et al., 1995). Shortly after the isolation of PCV2 in Canada, almost identical viruses were isolated from diseased pigs in North America (Allan et al., 1998a; Larochelle et al., 1999b), Europe (Allan et al., 1999b; Kennedy et al., 1998), and Asia (Kim and Chae, 2001b). Using monoclonal and polyclonal antisera, these isolated PCV2 have been shown to be antigenically distinct from PCV1 (Allan et al., 1998b; Ellis et al., 1998).

Serological surveys for PCV2 antibodies were undertaken and a significant number of pigs were found to be seropositive, with most of them showing no clinical signs compatible with PMWS (Labarque et al., 2000; Magar et al., 2000a; Magar et al., 2000b; Rodriguez-Arrioja et al., 2000; Sibila et al., 2004). Retrospective studies show that antibodies that react with PCV2 have been widespread in the pig population since at least 1969, indicating that PCV2 is not a new virus (Walker et al., 2000). The presence of PCV2 antibodies in European wild boars has been reported (Vicente et al., 2004). Because of a slight degree of antigenic cross-reactivity between PCV1 and PCV2 isolates (Allan et al., 1998b), it is possible that the
high seroprevalence rate of antibodies reported to PCV1 may be due to antibodies cross-reactive with PCV2.

PCV2 seroconversion shows a pattern that consists of declining of colostral antibodies during the lactation and nursery periods, with the lowest levels typically occurring at the end of the nursery period, and active seroconversion of most pigs occurring during the grower period as determined by an immunoperoxidase monolayer assay (IPMA) or ELISA tests (Rodriguez-Arrioja et al., 2002). It has been reported that following experimental infection of the piglets with a cell culture isolate of PCV2, the piglets with passively derived maternal antibodies from sows with high levels of PCV2 antibodies performed significantly better in terms of weight gain, clinical scores and disease compared to piglets derived from sows with low PCV2 antibodies, indicating maternal antibody plays an important role in protecting piglets from developing PMWS (Allan G.M., 2002).

Previous studies demonstrated that the genome organization of PCV1 and PCV2 consists of two major open reading frames (ORFs), ORF1 and ORF2. ORF1 encodes the replication associated protein (Rep) and ORF2 encodes the major capsid protein (Cap) (Liu et al., 2001; Nawagitgul et al., 2000). Recently, several studies have suggested that the PCV2 Cap is the major immunogenic protein of the virus and the principal bearer of type-specific epitopes (Blanchard et al., 2003a; Liu et al., 2001). In contrast, the Rep appears to be only weakly immunogenic (Blanchard et al., 2003a)

Pathological studies

PCV1 is generally accepted to be a non-pathogenic virus (Allan et al., 1995; Tischer et al., 1986). Experimental infection of pigs with PCV1 at various times after birth did not produce clinical disease (Allan et al., 1995; Tischer et al., 1986). In contrast, PCV2 has been
directly associated with a number of clinical conditions including PMWS, porcine dermatitis and nephropathy syndrome (PDNS), porcine respiratory disease complex (PRDC), enteritis, reproductive failure, and congenital tremor type All (Allan and Ellis, 2000; Segales et al., 2004b).

Viremia caused by PCV2 appears to be both cell-free and cell-associated, but the cell-associated form is much more apparent and long-lasting (Pensaert et al., 2004). It is of interest that the cell-associated viremia is present concomitantly with IPMA antibodies (Pensaert et al., 2004). In addition, the presence of PCV2 DNA in tissues from experimentally infected pigs at 125 days post-inoculation has been demonstrated, suggesting that there is a persistent infection by PCV2 (Bolin et al., 2001).

PCV2 has been shown to be transmissible by co-mingling animals from affected herds and naive animals (Balasch et al., 1999). Studies have shown that PCV2 can be detected by PCR in nasal, tonsillar, bronchial, urinary and fecal samples in a large proportion of pigs with and without PMWS (Calsamiglia et al., 2002; Shibata et al., 2003; Yang et al., 2003). Based on these studies, the potential route of exposure of young swine to PCV2 appears to be by either oronasal direct contact exposure or through fecal-oral transmission of PCV2 in feces. Viral transmission may also occur vertically, or by sexual contact because PCV2 has been detected in fetuses (O'Connor et al., 2001; West et al., 1999) and in semen (Larochelle et al., 2000). There has been no report of PCV2 infection from shared needles. However, several studies have demonstrated that PCV2 genomic DNA was infectious when injected intramuscularly into pigs (Fenaux et al., 2004a; Grasland et al., 2005; Roca et al., 2004), suggesting that blood transmission might be possible.
Recently, several studies were conducted to investigate PCV2 infection on other species. Data indicates that PCV2 can infect and cause PMWS at a higher incidence in European wild boar (*Sus scrofa*) subspecies compared to domestic swine (Ellis et al., 2003; Schulze et al., 2004). More recently, the detection of PCV1 and PCV2 DNA from porcine-derived commercial pepsin has been reported (Fenaux et al., 2004c). Though the PCV-contaminated pepsin lacks infectivity in PK-15 cells or *in vivo*, this finding raises the concern for potential human infection through xenotransplantation (Fenaux et al., 2004c).

**Genomic studies**

The genome sequences of a number of PCV1 (1759 bases) and PCV2 (1768 bases) isolates have been determined (Fenaux et al., 2000; Hamel et al., 1998; Meehan et al., 1998; Nayar et al., 1997). The overall DNA sequence identity within PCV1 or PCV2 isolates is greater than 90% (de Boisseson et al., 2004; Grierson et al., 2004; Larochelle et al., 2003; Mankertz et al., 2000; Meehan et al., 1998), while the identity between PCV1 and PCV2 isolates is 68%-76% (Fenaux et al., 2000; Hamel et al., 1998; Meehan et al., 1998; Nayar et al., 1997).

The Rep genes of PCV1 and PCV2 are highly conserved with 83% identity at the nucleotide sequence level and 86% at the protein level. In contrast, the Cap genes share only 67% identity at the nucleotide sequence level and 66% at protein level (Mankertz et al., 1998a; Mankertz et al., 1998b; Meehan et al., 1997). It has recently been reported that two amino acid mutations within the PCV2 capsid protein at passage 120 are responsible for the increased growth rate *in vitro* and for attenuation of the virus *in vivo* (Fenaux et al., 2004b).

PCV has an ambisense circular genome that encodes proteins by the encapsidated viral DNA, and by the complementary DNA of the replication intermediate synthesized in
the host (Tischer et al., 1982). The encapsidated viral DNA codes for the Rep-associated and non-structure (NS)-associated proteins while the complementary strand synthesized in the host codes for the Cap (Cheung, 2003a; Cheung, 2003c; Cheung and Bolin, 2002; Mankertz and Hillenbrand, 2001; Mankertz and Hillenbrand, 2002; Mankertz et al., 1998a; Mankertz et al., 1998b; Nawagitgul et al., 2000). A nuclear localization signal for the Cap has been identified (Liu et al., 2001). In order to express the Cap, it is believed that the complementary strand has to be synthesized through the intermediate double-stranded replication form (RF) (Tischer and Buhk, 1988).

During PCV1 replication in PK-15 cells, twelve virus-specific RNAs are synthesized. They include one capsid protein RNA, eight Rep-associated RNAs (rep, rep’, rep3a, rep3b, rep3c-1, rep3c-2, rep3c-3 and rep3c-4), and three NS-associated RNAs (Cheung, 2004). Nine RNAs were synthesized during productive PCV2 infection in PK-15 cells. They include the viral capsid protein RNA, a cluster of five Rep-associated RNAs (rep, rep’, rep3a, rep3b, and rep3c), and three NS-associated RNA (Cheung, 2003c). Corroborating earlier observations in PCV1 (Mankertz and Hillenbrand, 2001), the simultaneous expression of rep and rep’ proteins is essential for initiation of replication of PCV2 (Cheung, 2003b).

When the ratio of the two transcripts, rep and rep’, was measured following transfection of PK-15 cells with an infectious PCV1 clone, the amount of rep’ and rep were similar at the beginning of infection. Later, rep’ number increased and decreased again, indicating a delicately balanced regulation of rep gene expression (Mankertz et al., 1998a). It was shown that the rep/rep’ protein of PCV2 initiated replication of PCV1, as did the reciprocal combination, indicating that the cis- and trans-acting replication factors of the two viruses are functionally interchangeable (Mankertz et al., 2003).
PMWS and PCV2

Background of PMWS

PMWS was initially described in Saskatchewan, Canada in 1991 (Clark, 1997; Harding, 1996). The initial outbreaks of PMWS were in herds with high health status. Subsequently the syndrome was reported around the world, including; Europe, North America and Asia (Allan et al., 1999b; Allan et al., 1998b; Choi et al., 2000; Kiupel et al., 1998; Onuki et al., 1999; Saoulidis et al., 2002; Trujano et al., 2001). Affected herds are typically farrow-to-finish operations. However, PMWS has been described in almost all types of farms and farm size has ranged from 30- to 10,000-sow herds. The expression of the disease on an individual basis appears to be a key point in the clinical syndrome. PMWS affected pigs tend to die or to develop marked wasting within a few days of infection (Segales and Domingo, 2002). Morbidity and mortality associated with PMWS can be 4 to 10% and 70 to 80%, respectively, depending on the stage of the outbreak and the management style within the affected units (Segales and Domingo, 2002). In North America, PMWS usually results in low grade but persistent death losses. On rare occasions, epidemics resulting in a 3 to 4-fold increase in post-weaning mortality rates have been reported (Madec, 2000). Persistent, high mortality appears to be more common in several European countries, and may be associated with an epidemic form of PCV, co-existing diseases, factors of unique regional procedures (Madec, 2000). Environmental factors such as air drafts, overcrowding, poor air quality, co-mingling of age groups and other stressors may exacerbate the severity of the disease (Harding, 1998).
Clinical signs and pathology

The primary clinical signs of PMWS in affected pigs include wasting, dyspnea, and pallor. Other clinical signs including diarrhea, jaundice, coughing, pyrexia, central nervous signs and sudden death have also been reported (Allan and Ellis, 2000; Ellis et al., 1998).

Macroscopic lesions associated with PMWS are nonspecific and include enlarged lymph nodes such as the mesenteric, inguinal, and tracheobronchial lymph nodes. Non-collapsing, tan-mottled lungs, sometimes with marked interstitial edema has typically been associated with bronchopneumonia and has been associated with PMWS (Allan and Ellis, 2000; Rosell et al., 1999). PMWS affected animals may also have atrophic discolored livers, and/or multifocal white foci in the kidney’s cortices. Gastric ulceration of the pars oesophages can also be seen in PMWS affected pigs (Allan and Ellis, 2000; Rosell et al., 1999).

Microscopically, varying degrees lymphoid depletion together with histiocytic and/or multinucleate giant cell infiltration of lymphoid tissues is a unique lesion that characterizes PMWS. In a number of cases, basophilic intracytoplasmatic inclusion bodies containing PCV2 antigen or nucleic acid can be found in cells of the histiocytic lineage (Rosell et al., 1999). Sporadically, multifocal coagulative necrosis may be observed (Segales and Domingo, 2002). Other microscopic changes include multifocal lymphohistiocytic interstitial pneumonia, variable degrees of hepatitis, lymphocytic or lymphohistiocytic infiltration in the renal interstitium, and intestinal villous atrophy with variable sloughing and/or regeneration of glandular and cryptal epithelial cells (Allan and Ellis, 2000; Rosell et al., 2000a; Rosell et al., 1999).
Mild to moderate PMWS-like lesions can be observed in clinically normal pigs (Quintana et al., 2001), indicating that subclinical PCV2 infection occurs frequently in both PMWS and non-PMWS-affected farms (Rodriguez-Arrioja et al., 2000). Several studies have demonstrated a correlation between the amount of viral antigen or genetic material detected in the tissue and the severity of the lymphoid depletion. However, no correlation has been found between the severity of PMWS lymphoid lesions and the severity of lesions in other tissues such as lung, liver or kidney (Chianini et al., 2003; Darwich et al., 2002; Rosell et al., 1999).

**Experimental reproduction of PMWS**

Experimental studies with PCV2 have primarily focused on reproducing PMWS. Initial experimental trials using tissue homogenates or PCV2 isolated and propagated in cell culture, reproduced PMWS-like lesions of slight to moderate intensity (Allan et al., 1999a; Balasch et al., 1999; Ellis et al., 1999; Kennedy et al., 2000; Krakowka et al., 2000; Magar et al., 2000a; Pogranichnyy et al., 2000). However, no clinical wasting occurred. Later on, reproduction of the full spectrum of PMWS clinical and pathological outcome using PCV2 alone have been reported (Allan et al., 2002; Bolin et al., 2001; Harms et al., 2001). The infection of conventional segregated early weaned (SEW) pigs with an infectious DNA clone of PCV2, resulting in the hallmark lesions consistent with PMWS, further established PCV2 as the cause of PMWS (Fenaux et al., 2002).

Although severe PMWS has been reported to be induced with PCV2 alone, severe clinical PMWS has been more reliably reproduced in pigs co-infected with porcine parvovirus (PPV) (Kennedy et al., 2000; Krakowka et al., 2000), porcine reproductive and respiratory syndrome virus (PRRSV) (Harms et al., 2001; Rovira et al., 2002), *Mycoplasma*
hyopneumoniae (Opriessnig et al., 2004), or with the use of immunostimulating factors, such as keyhole limpet hemocyanin (KLH) emulsified in incomplete Freund’s adjuvant, vaccines, or non-specific immunomodulating drugs (Baypamun, Bayer, Leverkusen, Germany) (Allan et al., 2000; Krakowka et al., 2001; Kyriakis et al., 2002; Opriessnig et al., 2003). The use of these co-factors suggests that multiple factors may be needed to reproduce the full spectrum of clinical PWMS.

However, contradictory results from several studies on immunostimulation and the induction of PMWS have been reported (Ladekjaer-Mikkelsen et al., 2002; Resendes et al., 2004a). One study found that using KLH emulsified in incomplete Freund’s adjuvant in 3 week old conventional PCV2 infected pigs did not always result in clinical disease, though PMWS was produced in several pigs (Ladekjaer-Mikkelsen et al., 2002). A second study found that the use of a commercial adjuvant did not result in the development of PMWS in conventional pigs experimentally infected with PCV2, suggesting that not all conditions or adjuvants used in commercial vaccines are capable of triggering PMWS (Resendes et al., 2004a). Moreover, an investigation of the effect of immunosuppression on PCV2 infections using an immunosuppressive drug, dexamethasone (DEX) showed that a reduced number of CD4^+ T cells in DEX-treated piglets (with or without PCV2 infection) and PCV2 associated lesions and antigen was present in lymphoid tissues from the PCV2 and DEX treated group. These results again conflict with the hypothesis that immune stimulation may predispose pigs to PMWS and suggest that suppression of cell-mediated immunity may play a role in the etiology of PMWS (Kawashima et al., 2003).
Field observations

In addition to recognizing PCV2 as the cause of PMWS, studies have documented that PMWS-affected pigs in the field are typically concomitantly infected with other pathogens including PRRSV, PPV, swine influenza virus (SIV), pseudorabies virus (Ellis et al., 2000; Quintana et al., 2001; Rodriguez-Arrioja et al., 1999; Rosell et al., 1999; Segales et al., 2000), *Mycoplasma hyopneumoniae* and a variety of other bacterial pathogens, notably *Streptococcus suis*, *Bordetella bronchiseptica Pasteurella multocida*, *Actinobacillus pleuropneumoniae*, and *Staphylococcus* sp. (Kim and Chae, 2004a; Madec, 2000; Pallares et al., 2002; Pogranichniy et al., 2002; Quintana et al., 2001; Rodriguez-Arrioja et al., 1999; Segales et al., 2002). The presence of these secondary pathogens suggests that they may play an important role in inducing PCV2 infection to progress to PMWS (Segales and Domingo, 2002).

However, a comparative serological and virological study was performed in pigs from herds with and without PMWS in Quebec (Larochelle et al., 2003). The serum was evaluated for the presence of antibodies to PCV2 and PRRSV by IFA and ELISA, respectively. A PCR assay was used to detect the nucleic acid of PCV2, PRRSV, and PPV. The PCV2 strains detected in the herds with or without PMWS were sequenced and their genomes compared. The results of this study demonstrated that the development of PMWS could not be linked to co-infection with either PRRSV or PPV or to particular genomic differences between PCV2 strains (Larochelle et al., 2003). These results further complicate our understanding of the pathogenesis of PCV2 infection and PMWS under field conditions.
**Immunological aspects**

The lack of response to antibiotic therapy in systemic and/or pulmonary bacterial infections in PMWS affected farms (Segales et al., 2004a; Segales et al., 1997) as well as infections with unusual swine pathogens, such as *Pneumocystis carinii*, *Chlamydia* spp., *Aspergillus* spp and *Cryptosporidium parvum*, suggests an immunosuppressive feature associated with PMWS (Carrasco et al., 2000; Clark, 1997; Darwich et al., 2004; Ellis et al., 1998; Nunez et al., 2003; Segales et al., 2004a). Furthermore, pathologic, immunohistologic and flow cytometric studies also suggest that pigs with PMWS are immunosuppressed.

Lymphocyte depletion of follicular and interfollicular areas together with macrophage infiltration of lymphoid tissues is a unique feature of lesions observed in pigs with PMWS (Clark, 1997; Darwich et al., 2002; Quintana et al., 2001; Rosell et al., 1999; Sarli et al., 2001). These findings are highly correlated with a decrease in circulating B and T cells (Darwich et al., 2002; Nielsen et al., 2003; Segales et al., 2000) as well as a reduction in these cell types in lymphoid tissues (Chianini et al., 2003; Sarli et al., 2001; Shibahara et al., 2000). Concurrently, an increase of macrophages/monocytes occurs in both the peripheral blood and lymphoid tissues (Segales et al., 2001). These changes have been observed in pigs both naturally and experimentally infected with PCV2 exhibiting PMWS. The altered populations of cells associated with the immune system response both in blood and tissues suggests that actively infected pigs have a transient inability to mount effective immune responses.

To understand the mechanism of lymphoid depletion in association with PCV2 infection and PMWS, the role of immune cell proliferation and apoptosis has been assessed in diseased pigs. A high number of apoptotic B lymphocytes and a decrease in the number of...
B lymphocytes with the confinement of PCV2 antigen and virions in the apoptotic bodies phagocytosed by macrophages was observed in the lymphoid tissues of pigs manifesting clinical signs of PMWS, suggesting that PCV2 induced apoptosis and resulted in B lymphocyte depletion (Shibahara et al., 2000). However, recent studies appear to contradict these earlier results. One study found that reduced levels of apoptosis were in the B cell areas of lymphoid tissues of PMWS affected pigs compared to healthy non-PCV2 infected pigs (Resendes et al., 2004b). Another study detected PCV2 and apoptotic cells in PMWS affected pigs with differing severity of lesions using in situ hybridization (ISH) and cleaved caspase-3 (CCasp3) immunohistochemical assay (Resendes et al., 2004c). Results from this study found that the apoptotic rate in lymphoid tissues was correlated inversely with the viral load in serum and the severity of lesions, indicating that apoptosis is not a significant feature in PMWS lymphoid lesion development (Resendes et al., 2004c). The third study evaluated the proliferative activity and apoptosis of lymphocytes to determine potential mechanisms for the pathogenesis of cell depletion in lymph nodes in spontaneous cases of PMWS. The apoptotic and proliferative activity were investigated (Mandrioli et al., 2004). The results of this study suggested that decreased cell proliferation and not increased apoptosis seemed to be the most important variable leading to cell depletion in PMWS lymphoid tissues (Mandrioli et al., 2004). The fourth study examined the colocalization of PCV nucleocapsid and terminal deoxynucleotidyld transferase (TdT)-mediated dUTP-nick end labeling (TUNEL) in lymphoid tissues and livers of gnotobiotic pigs with PMWS (Krakowka et al., 2004). The data from this study also suggested that apoptosis is not the primary mechanism of lymphoid depletion and hepatocyte loss in PMWS. Apoptosis associated with systemic viral diseases may be attributable to pyrexia rather than direct or indirect effects of PCV2 on target cells.
However, all these findings are based on the studies of PMWS affected pigs. The effect of PCV2 on the immune cell proliferation and apoptosis at early stage of infection remains unknown.

The changes of cytokine RNA and protein expression were assessed to study the pathogenesis of PCV2 infection and PMWS. A study demonstrated that pigs with PMWS have altered cytokine mRNA expression patterns in affected lymphoid tissues (Darwich et al., 2003b). These alterations consist of an over expression of interleukin (IL)-10 mRNA in thymus and of interferon (IFN)-gamma mRNA in tonsils. The IL-10 mRNA over expression has been associated with thymic depletion and atrophy in the diseased animals (Darwich et al., 2003b). Reduced levels of IL-2 mRNA expression in the spleen, IL-4 in tonsils and lymph nodes, IL-12p40 in both spleen and inguinal lymph nodes and IFN-gamma and IL-10 in inguinal lymph nodes have also been reported (Darwich et al., 2003b). Another study investigated the systemic expression of relevant cytokines at both the mRNA and protein levels in PBMCs of pigs suffering from natural PMWS and in clinically healthy pen-mates (Sipos et al., 2004). Results of this study indicated that IL-1 alpha and IL-10 mRNA levels were notably increased in the affected pigs, whereas IL-2 and IL-2 R alpha (CD25) mRNA levels tended to be down-regulated. In addition, IL-8, tumor necrosis factor (TNF)-alpha and IFN-gamma mRNA expressions appeared to be slightly increased. Moreover, the detection of intracellular cytokines at the protein level revealed an increase in levels of IL-1 beta, IL-2, and IL-6, while IL-12 and TNF-alpha expressions were not affected, and IFN-gamma levels were slightly decreased in the diseased animals (Sipos et al., 2004).

The in vitro study of PCV2 infection of PBMCs from healthy and diseased pigs revealed substantial and specific affects on the function of PBMCs from PMWS pigs in terms
of cytokine release (Darwich et al., 2003a). By testing the supernatants from cell cultures using capture ELISAs, this study found that mononuclear cells from pigs with PMWS were less able to produce IFN-gamma when superantigens were added to the cultures, and IL-2, IL-4 when a T-cell mitogen, phytohemagglutinin (PHA) or superantigens were added to the cultures. However, in contrast to PBMCs from healthy pig, cells from PMWS affected pigs produced some IFN-gamma or IL-10 when recall PCV2 antigens were added for 24 hours (Darwich et al., 2003a). Furthermore, PCV2 alone appears to alter the cytokine secretion of PBMCs from healthy and diseased pigs by showing a decrease in IL-2 secretion and IFN-gamma when PBMCs exposed to the virus and then challenged with PHA (Darwich et al., 2003a). Although these studies showed the alteration of cytokine profile in PBMCs and lymphoid tissues from PMWS affected pigs in vivo or in vitro, there is still lack of study on the changes of cytokine RNA and protein expression within specific immune cell populations.

Granulomatous infiltration of lymphoid tissues is one of the characteristics for PMWS (Rosell et al., 1999). Since the recruitment of monocytes into an area of inflammation by the secretion of chemokines is an important step in this process, the correlation of chemokine expression and PCV2 infection has also been studied. The chemokine family can be divided into two major classes, CC and CXC, on the basis of differences in the position of cysteines within a conserved four-cysteine motif (Oppenheim et al., 1991; Rollins, 1997). The CC chemokines, such as monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein-1 (MIP-1) have powerful chemoattractant and activator properties for monocytes (Oppenheim et al., 1991; Rollins, 1997; Wolpe et al., 1988), whereas the CXC chemokines, such as IL-8, are more important in the attraction of neutrophils (Oppenheim et
al., 1991; Rollins, 1997). A positive correlation between PCV2 and MCP-1 and MIP-1 expression in serial sections from the lymph nodes of piglets inoculated intranasally with PCV2 was demonstrated, suggesting an up regulation of MCP-1 and MIP-1 expression by mononuclear cells in response to PCV2 (Kim and Chae, 2003; Kim and Chae, 2004b). It has also been reported that IL-8 mRNA levels are increased in tissues with slight or moderate lesions and small amounts of virus while it is reduced in tissues with severe lesions (Darwich et al., 2003b). However, this is in contrast to other investigations which did not find altered levels of IL-8 mRNA in granulomatous lesions of lymph nodes from pigs with naturally occurring PMWS, but found high levels of MCP-1 (Kim and Chae, 2003).

**PCV2 infection and replication**

Studies consistently demonstrate that PCV2 nucleic acid or antigen in PMWS-affected pigs is primarily in the cytoplasm of histiocytes, multinucleate giant cells and other monocyte/macrophage lineage cells such as alveolar macrophages, Kupffer cells and follicular dendritic cells of lymphoid tissues by ISH and IHC (Allan and Ellis, 2000; Chianini et al., 2003; Ellis et al., 1998; Gilpin et al., 2003; Rosell et al., 1999). Therefore, the monocyte/macrophage lineage cells were thought to be the primary cell type for PCV2 replication. It has also been reported that PCV2 is sporadically detected in the cytoplasm of renal and respiratory epithelial cells, vascular endothelium cells, lymphocytes, pancreatic acinar and ductular cells and in the nuclei of monocyte/macrophage cells, smooth muscle cells, hepatocytes and enterocytes (Allan et al., 1999a; Ellis et al., 2000; Kennedy et al., 2000; Krakowka et al., 2000; McNeilly et al., 1999; Rosell et al., 2000a; Rosell et al., 1999; Sanchez et al., 2004; Shibahara et al., 2000; Sirinarumitr et al., 2000).
The replication of PCV2, as for other circoviruses, depends on the cellular polymerases present in the nucleus of actively dividing cells because PCV2 does not encode its own polymerase (Tischer et al., 1987). Although macrophages do not divide, it has been shown that they can express high DNA polymerase activity in response to damage to their DNA (Terai et al., 1991; Williams et al., 2002), an event which might be misused by PCV2 to complete its replication in these cells (Meerts et al., 2005). However, several in vitro studies have not found evidence of PCV2 replication in monocyte/macrophage lineage cells. In one in vitro study, PCV2 antigen was detected in the cytoplasm of monocytes, pulmonary alveolar macrophages (PAMs) and monocyte-derived macrophages but the virus did not replicate in PAMs, which suggested that in vivo, monocytic cells may not the primary cells that support PCV2 replication (Gilpin et al., 2003). Another study found no evidence for PCV2 replication in dendritic cells (DCs), but the virus appeared to persist in them without loss of infectivity nor the induction of cell death, demonstrating that PCV2 can persist in DCs in the absence of virus replication or degradation (Vincent et al., 2003). Moreover, there was no modulation of DC surface markers including the major histocompatibility complex class I and class II, CD80/86, CD25, CD16, or CD14. Infected DCs did not transmit virus to syngeneic T lymphocytes, even when the latter were activated (Vincent et al., 2003).

Recently, the replication kinetics of PCV2 in PAM and fetal cardiomyocytes (FCM), two target cells in vivo, was compared with replication in PK-15 cells (Meerts et al., 2005). Results from this study showed that PCV2 replicates in PK-15 cells, but not in either the PAM or FCM cultures (Meerts et al., 2005). Viral proteins were visualized in the nucleus of FCM and PAM later (48 hours post-infection (HPI)) than in PK-15 cells (between 12 and 24 HPI) and in a lower percentage of cells. In PAMs, two out of five tested pigs had no apparent
nuclear localized antigens, whereas in the other three pigs viral antigens were observed in up to 20% of the cells, suggesting clear differences in susceptibility of PAM from different pigs.

Modification of PCV2 target cells during fetal to postnatal development in pigs has been examined (Sanchez et al., 2003). PCV2 inoculation was performed in fetuses in utero at various days of gestation and in piglets at 1 day of age. The study revealed that PCV2 target cells change from cardiomyocytes, hepatocytes and macrophages during fetal life to only macrophages postnatally (Sanchez et al., 2003). In addition, less than 5% of any of the cell types contained PCV2 genome in the nucleus (Sanchez et al., 2003). An apparent hepatotropism for PCV2 has also been observed with cyclosporine immunosuppressed PCV2-infected gnotobiotic piglets (Krakowka et al., 2002). To date, the cell tropism for PCV2 infection and replication remains unknown. In our studies, we assessed PCV2 replication in immune cell populations during virus active replication stage in vitro and in vivo. We demonstrated that lymphocytes are the important cells for PCV2 replication compared to monocytes.

**Diagnosis**

The diagnosis of PMWS must meet three criteria: (i) the presence of compatible clinical signs, (ii) the presence of characteristic microscopic lesions, and (iii) the presence of PCV2 antigen or nucleic acids within the lesions. The three criteria considered separately are not diagnostic of PMWS (Allan and Ellis, 2000; Chae, 2004).

Several methods such as virus isolation, IFA, ISH, IHC and PCR have been developed to detect PCV2 in tissues or serum samples (Allan et al., 1998b; Choi et al., 2000; Ellis et al., 1998; McNeilly et al., 1999; Quintana et al., 2001; Rosell et al., 1999; Tischer et al., 1987). Among them, ISH and IHC are the tests most routinely performed and are
typically considered the gold standard to establish a PMWS diagnosis (Choi et al., 2000; Ellis et al., 1998; McNeilly et al., 1999; Rosell et al., 1999). It has been reported that not all monoclonal and polyclonal antibodies to PCV2 are suitable for use in IHC due to the cross-linking effects of formalin fixation that renders certain epitopes undetectable (Haines and Chelack, 1991). In contrast, ISH is less susceptible to structural alteration from formalin fixation (Choi and Chae, 1999; Kim and Chae, 2001a) and allows the differentiation between PCV1 and PCV2 in formalin-fixed, paraffin-wax-embedded tissues with a nonradioactive digoxigenin-labeled probe (Kim and Chae, 2001a; Kim and Chae, 2002). A strong correlation has been observed between the amount of PCV2 nucleic acid or antigen, the severity of microscopic lymphoid lesions and the clinical outcome (Kennedy et al., 2000; Quintana et al., 2001).

PCR has been shown to be the most sensitive technique to detect PCV2 and a useful technique to test potential routes of viral excretion (Allan et al., 1999b; Calsamiglia et al., 2002; Choi et al., 2000; Kim and Chae, 2001b; Kim et al., 2001; Larochelle et al., 1999a; Mankertz et al., 2000; Quintana et al., 2001; Shibata et al., 2003; West et al., 1999). However, as PCV2 is so common within the swine population, its detection by PCR in serum or inguinal lymph node has a lower correlation with the typical microscopic lesions of the syndrome (Calsamiglia et al., 2002). As a result, it is felt that qualitative PCR is not suited for PMWS diagnosis (Chae, 2004).

In addition to qualitative PCR assays, quantitative real-time PCR assay has been described and mostly applied to serum samples (Ladekjaer-Mikkelsen et al., 2002; Olvera et al., 2004; Rovira et al., 2002). Due to the correlation between PCV2 levels and the severity of clinical disease, a quantitative PCR represents a useful (but expensive) technique for use
to diagnose PMWS (Liu et al., 2000; Rovira et al., 2002). Recently, a TaqMan-based real-time PCR for quantitation of PCV2 in tissue and plasma/serum samples has been established (Brunborg et al., 2004). In supporting previous findings that the PCV2 load in the animal is correlated with disease, a significant difference in estimated viral load between healthy (less than $10^6$ PCV2 genome copies per ml serum or 500 ng tissue sample) and PMWS pigs (above $10^7$ PCV2 genome copies per ml serum or 500 ng tissue sample) was found in samples from mesenteric lymph nodes as well as serum/plasma (Brunborg et al., 2004). Furthermore, the estimated viral load in tissue samples from PMWS pigs was related to the IHC results, with lymph nodes, ileum, and tonsil giving both high levels of virus and a high amount of staining by IHC (Brunborg et al., 2004). Interestingly, it was found that for most tissues an estimated minimum viral level of $10^8$ PCV2 genome copies per 500 ng DNA was required in order to give a visible staining by IHC. Even with viral load as high as $10^8$ PCV2 genome copies per 500 ng DNA, no visible viral antigen staining was observed in the kidneys and myocardium of PMWS pigs (Brunborg et al., 2004). In agreement with this finding, with similar levels of PCV2 DNA detected by real-time PCR, we observed PCV2 antigen in lymph nodes, spleen, tonsil, thymus, liver and lung from infected pigs but not in kidney by IHC in our study of PCV2 distribution and replication in tissues and immune cells in early infected pigs.

Several serological techniques such as IFA, IPMA, competitive ELISA and indirect ELISA assays have been used to detect antibodies to PCV2 (Allan et al., 1998b; Blanchard et al., 2003b; Ellis et al., 1998; Liu et al., 2004; Nawagitgul et al., 2002; Rodriguez-Arrioja et al., 2002; Walker et al., 2000). Because PCV2 is ubiquitous within the swine population and
the seroconversion pattern is relatively similar between PMWS affected and non-affected farms, serological techniques cannot be used to diagnose disease.

**Prevention and control**

Treatment and control of PMWS has primarily been focused on providing good production practices and eliminating potential triggering factors that induce the immune stimulation suspected to cause disease. Although little research has been undertaken in this area, maintaining ideal pen density, age segregation and all-in–all-out pig flow with the timely removal of sick animals has been widely recommended (Allan and Ellis, 2000; Segales and Domingo, 2002). A sero-therapy technique has been used with success in Europe, but has not been widely accepted in other parts of the world (Ferreira D., 2001).

Several commercial vaccines for sows and pigs are currently in the licensing phase in the United States and Europe. Although no PCV2 vaccine is currently available, an ORF1/ORF2-DNA vaccine and an ORF1/ORF2-subunit vaccine have been developed and tested experimentally (Blanchard et al., 2003a; Kamstrup et al., 2004; Truong et al., 2001). ORF2-based vaccine candidates have been shown to induce specific antibody responses and good protection when comparing vaccinated and non-vaccinated piglets following experimental PCV2 challenge (Blanchard et al., 2003a; Kamstrup et al., 2004). A chimeric PCV1-2 live virus (with the immunogenic capsid gene of PCV2 cloned into the backbone of PCV1) as well as the chimeric PCV1-2 infectious DNA clone has also been suggested as a potential vaccine (Fenaux et al., 2004a)

**Other PCV2 associated diseases**

Although PCV2 has primarily been associated with PMWS, it has also been implicated in a number of disease syndromes including PDNS, PRDC, enteritis, reproductive
failure, and congenital tremors type AII (Allan and Ellis, 2000; Segales et al., 2004b).

However, the role of PCV2 in these conditions has not been fully elucidated and in some of
these conditions its role in disease remains controversial (Kennedy et al., 2003).

Porcine dermatitis and nephropathy syndrome

Porcine dermatitis and nephropathy syndrome (PDNS) associated with PCV2
infection was originally described in the UK (Smith et al., 1993). Affected pigs were in both
the nursery and grow-finish stages and its appearance within a herd has been generally
sporadic (Gresham et al., 2000; Rosell et al., 2000b; Thomson et al., 2000). The mortality
associated with PDNS ranges from 10 to 25% in Europe, whereas it is still infrequently
recognized in North America, where typically less that 0.5% of pigs are affected (Meehan et
al., 1998; Rosell et al., 2000b). PDNS-affected pigs demonstrate anorexia, depression,
prostration, a stiff-gait or reluctance to move, and occasionally mild pyrexia. The most
common clinical signs are the development of round or irregular shaped, red to purple skin
lesions that coalesce to form larger patches and plaques. These lesions are located primarily
on the hind limbs and in the perineal area (Helie et al., 1995; Segales et al., 1998). At post­
mortem, the kidneys are enlarged, pale and often covered with petechial hemorrhages.
However, not all affected pigs show macroscopic kidney or skin lesions (Segales et al.,
2004b). Major histopathological findings include necrotizing and fibrinous
glomerulonephritis and systemic necrotizing vasculitis (Segales et al., 1998; Thibault et al.,
1998). Recently, it was demonstrated that PDNS affected pigs had higher mean PCV2 serum
antibody titers than the antibody titers in pigs without clinical signs of PDNS, suggesting the
causative physiological basis for PDNS may be the excessive levels of PCV2 antibodies
(Wellenberg et al., 2004). These microscopic features, together with the presence of
immunoglobulin and complement components in the damaged vessels and glomeruli, suggest a type III hypersensitivity reaction as a possible mechanism for disease (C.O. Duran, 1997; Helie et al., 1995; M.A. Sierra, 1997; R. Drolet, 1999; Wellenberg et al., 2004). PCV2 antigens and/or nucleic acids have been found in the tissues of pigs with PDNS (Rosell et al., 2000b). However, evidence suggests that other pathogens such as *P. multocida* (Lainson et al., 2002; Thomson et al., 2001), or PRRSV (Choi and Chae, 2001; Rosell et al., 2000b; Thibault et al., 1998) may also be the potential antigen that results in PDNS. Many herds in Europe report the simultaneous or closely timed occurrence of PMWS and PDNS (Rosell et al., 2000b; Segales et al., 1998). Unlike PMWS, however, PDNS has not been experimentally reproduced so the etiology of PDNS remains in question.

**Porcine respiratory disease complex**

Porcine respiratory disease complex (PRDC) is characterized by slow and uneven growth, reduced feed intake, higher feed to gain conversion rates, cough and clinical pneumonia (Halbur PG, 1998; Thacker, 2001). This condition often occurs when pigs are 16-20 weeks of age. Major pathogens involved in PRDC include PRRSV and *M. hyopneumoniae*. However, a number of other bacterial and viral pathogens have also been detected in PRDC outbreaks (Thacker, 2001). Studies using IHC have been conducted on diagnostic submissions of cases of PRDC in the midwestern United States and have documented a high prevalence of co-infection with PRRSV in cases of PCV2-associated pneumonia, with a predominance of PCV2 antigen in the dually infected lungs (Harms et al., 2001). Co-infection of PCV2 with SIV and *M. hyopneumoniae* was also reported in those studies. In many of the cases, no clear-cut clinical differences exist between PRDC and PMWS cases. Macroscopic lung lesions caused by PCV2 are virtually indistinguishable from
those induced by PRRSV or even systemic bacterial infections such as salmonellosis (Harms, 2000). The microscopic lesions of PCV2-infected pigs in the lung (interstitial pneumonia) are not specific for the organism. However, the presence of airway epithelial sloughing and mucosal or submucosal replacement by fibroplasia and lympho-histiocytic cell infiltrations throughout all lung lobes are considered characteristic of PCV2 infection (Clark, 1997). Although it is not clear whether PCV2 plays the role of a primary, synergistic, secondary, or opportunistic pathogen, demonstration of unique lung lesions associated with PCV2 antigen suggests that PCV2 is playing an important role in PRDC in the U.S.

**Enteritis**

The presence of PCV2-associated enteritis has been reported in pigs with a history of diarrhea with or without wasting. The lesions consist of granulomatous inflammation affecting Peyer’s patches, characterized by infiltration of epithelioid macrophages and giant multinucleated cells. Large, multiple, basophilic or amphophilic, grape-like intracytoplasmic inclusion bodies are often observed in the cytoplasm of histiocytic cells and giant multinucleated cells. In addition, PCV2 nucleic acid is present in the cytoplasm of histiocytes and giant multinucleated cells in the Peyer’s patches. The presence of diarrhea and granulomatous enteritis, and abundant PCV2 DNA associated with the microscopic lesions is suggestive of PCV2-associated enteritis. This finding suggests that PCV2-associated enteritis may be a distinct clinical manifestation of PCV2 infection (Kim et al., 2004a).

**Reproductive failure**

There have been a number of reports of PCV2-associated reproductive failure (Kim et al., 2004b; Ladekjaer-Mikkelsen et al., 2001; O'Connor et al., 2001; West et al., 1999). Consistent clinical signs on infected farms include abortions and elevated rates of stillbirth
and fetal mummification. The predominant lesion is a non-suppurative to necrotizing or fibrosing myocarditis in the fetuses. Variable amounts of PCV2 antigen are present in fetal tissues, and in the cardiac lesions of affected piglets. Studies in experimentally infected pregnant sows have shown that PCV2 infection alone can produce severe myocardial lesions in infected pig fetuses (Sanchez, 2001). The examination of stillborn fetuses collected from farms with prolonged histories of reproductive problems confirms previous findings that transplacental infection of PCV2 occurs in the field (Farnham et al., 2003). Recently, direct intra-fetal inoculation of PCV2 at different stages of gestation and collection of fetuses 21 days later showed that the virus replicates in fetal tissues, particularly in the heart. Fetuses inoculated at 57 days of gestation had the highest virus titers and no antibodies while antibodies and lower virus titers were detected in fetuses inoculated at 75 and 92 days of gestation. Inoculation at 57 and 75 days of gestation and collection of the piglets at the end of pregnancy demonstrated that intrauterine spread had occurred to fetuses adjacent to the inoculated ones and that fetal death also occurred in the presence of antibodies. However, the pregnancy was not interrupted (Pensaert et al., 2004). Another study reported that 10 of 14 pregnant sows inoculated with PCV2 directly into one of the fluid-filled fetal compartments at mid- and late-gestation, respectively, had dead and/or stillborn piglets at the end of gestation (Yoon et al., 2004). In addition, PCV2 infection was evident by PCR and/or IHC in those sows while five of six sham-inoculated control sows had no or minimal adverse affects from in utero injection (Yoon et al., 2004). More recently, it has been reported that six sows intranasally inoculated with PCV2 3 weeks prior to the expected farrowing date had abortions and premature farrowing. In contrast, two uninfected negative control sows remained clinically healthy and farrowed at the normal time. PCV2 antigen and DNA were
detected in the lymph nodes, spleen, thymus, lung, tonsil and liver from both stillborn and
liveborn piglets from the infected sows (Park et al., 2005). This experiment suggests that
PCV2 is capable of crossing the placenta, replicating primarily in lymphoid tissues, and
inducing reproductive failure in sows. However, in a retrospective examination of diagnostic
tissues submitted to the University of Saskatchewan between 1995 and 1998, neither PCV1
or PCV2 antigen, nor PCV2 nucleic acid was detected by IHC or PCR, respectively, in any
reproductive tissues (Bogdan et al., 2001).

**Congenital tremors type AII**

Congenital tremors (CT) in pigs is characterized by a tremor of the head and limbs in
new-born pigs (Edwards, 1999). Its subtype AII has traditionally been associated with an
unidentified virus, but several reports from the U.S. have suggested that PCV (Hines, 1994)
or more precisely, PCV2 (Stevenson et al., 2001) is the causal agent of this CT subtype. It
has been reported that PCV nucleic acid was detected in neural tissues and liver of both CT-
affected and normal pigs (Stevenson et al., 2001). However, recent work performed in
Europe on cases of CT subtype AII did not find circovirus nucleic acid or antigen in any CT
piglets (Kennedy et al., 2003).
CHAPTER 2. DEVELOPMENT OF A REVERSE TRANSCRIPTION-PCR ASSAY TO DETECT PORCINE CIRCOVIRUS TYPE 2 TRANSCRIPTION AS A MEASURE OF REPLICATION

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Abstract

Porcine circovirus type 2 (PCV2) is a non-enveloped, single-stranded, circular DNA virus. *In situ* hybridization and PCR assays have detected PCV2 DNA in multiple organs and cell types from infected pigs; however, it is not clear if this represents replicating virus or virion DNA. We describe the development of a single-tube RT-PCR assay to differentiate PCV2 replication products and viral DNA. Primers targeted to the open-reading frame 2 (ORF2) of PCV2 were designed to amplify both virus DNA (984 bp) and the spliced capsid Cap mRNA (594 bp). The 984 bp fragment, but not the 594 bp fragment, was amplified from PCV2 stock, confirming that the spliced Cap mRNA was not present in the PCV2 stock. The 594 bp fragment was amplified from DNase-treated RNA extracted from PCV2-infected PK-15 cells, and was detected as early as 14 hours post-infection. No products were amplified from either the PCV1 stock or PCV1-infected PK-15 cells, or from cells infected with

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Porcine circovirus (PCV) is a small, non-enveloped, single-stranded, circular DNA virus with a genome of approximately 1.7 kb belonging to the genus *Circovirus* of the *Circoviridae* family. It has an ambisense genome that encodes proteins by the encapsidated viral DNA and the complementary DNA of the replicative intermediate synthesized in the host. Two genotypes of PCV have been identified. PCV type 1 (PCV1), considered non-pathogenic, is a common contaminant of the porcine kidney cell line PK-15 (CCL-33) (Tischer et al., 1982). Serological surveys have demonstrated a high prevalence of anti-PCV1 antibodies in the swine population but no disease has been correlated with PCV1 infection (Tischer et al., 1986; Mahe et al., 2000). A newly emerged disease designated postweaning multisystemic wasting syndrome (PMWS) was first identified in a swine herd in Canada in 1991 (Ellis et al., 1998; Harding, et al., 1997). The cause of PMWS was attributed to a new PCV variant designated PCV type 2 (PCV2). Since then, PCV2 has been reported in many countries (Allan et al., 1999; Fenaux et al., 2000) and is associated with PMWS, respiratory disease, reproductive failure and a variety of other disease manifestations (Segales et al., 2004). PCV2-associated PMWS is characterized by depletion of lymphocytes in the lymphoid follicles and their replacement by macrophages. Variable amounts of granulomatous inflammation are also observed in other tissues. The severity of the
microscopic lesions and the amount of viral antigen in the lesions closely correlates with the severity of clinical disease (Quintana et al., 2001; Rosell et al., 1999). PCV2 antigen and DNA can be detected using immunohistochemistry (IHC), in situ hybridization (ISH) and polymerase chain reaction (PCR) from multiple organs and multiple cell types from infected pigs; however, the target cell for viral replication has not been definitively identified. To better understand specific cell populations that support viral replication in vitro and in vivo, an assay that discriminates between replicating and non-replicating virus would be useful.

The genome organization of PCV1 and PCV2 consists of two major open reading frames (ORFs), ORF1 and ORF2. ORF1 encodes the replication associated protein (Rep) and ORF2 encodes the major capsid protein (Cap) (Nawagitgul et al., 2000; Liu et al., 2001). The overall DNA sequence identity within PCV1 or PCV2 isolates is greater than 90%, while the identity between PCV1 and PCV2 isolates is 68-76% (Fenaux et al., 2000; Hamel et al., 1998). The Rep genes of PCV1 and PCV2 are highly conserved, with 83% identity at the nucleotide sequence level, whereas the Cap genes share only 67% identity (Mankertz et al., 1998). Transcriptional analysis of PCV2 identified one capsid mRNA and a cluster of five Rep-associated RNAs, which increase in a time dependent manner during PCV2 replication in PK-15 cells (Cheung, 2003). Interestingly, the capsid mRNA contains a splice junction at nucleotides 361 and 1737 (Cheung, 2003). Using primers designed to amplify across the Cap mRNA splice junction, we developed a PCV2-specific reverse transcription polymerase chain reaction (RT-PCR) assay that can differentiate between PCV2 replication products and virus DNA.

ORF2 sequences from PCV1 (GenBank Accession No. AY184287) and PCV2 (GenBank Accession No. AF264042) were aligned and PCV2-specific primers were
designed to amplify across the Cap mRNA splice junction. Amplification with the forward primer, CapF: 5'-GGAGTCAAGAACAGGTTTGCG-3' (nucleotides 232-254) and the reverse primer, CapR: 5'-AGACTCCGCTCTCGAAAG-3' (nucleotides 1196-1216) resulted in amplification of two different sized product, a 984 bp product amplified from virus DNA and a 594 bp product amplified from spliced Cap mRNA (Fig. 1). To determine if the RT-PCR assay could distinguish between PCV1 and PCV2, nucleic acid was isolated from either PCV1 that originated from the ATCC PK-15 cell line (PCV1 stock) (Tischer et al., 1986) and PCV2 isolated from splenic tissue from a pig with naturally occurring PMWS (isolate ISU-40895) (PCV2 stock) (Fenaux et al., 2002). The DNA was isolated using QIAamp DNA Mini kit (QIAGEN) according to the manufacturer’s instructions, while total RNA was isolated using the E.Z.N.A.® total RNA kit (Omega Biotek, Doraville, GA) and treated with DNA-free™ (Ambion, Austin, TX). All samples were quantified and the purity determined using a spectrophotometer. The O.D.260/O.D.280 ratios were above 1.80 corresponding to 90–100% pure nucleic acid. The RT-PCR assay was performed in a Mastercycler® (Eppendorf, Westbury, NY) using the Access RT-PCR System (Promega, Madison, WI) according to the manufacturer’s instructions. The RT-PCR reaction was performed in 50 µl total volume per sample with 0.2 mM dNTPs, 1x AMV/Tfl reaction buffer, 1 mM MgSO4, 1 µM of each primer, 0.1 U/µl of AMV reverse transcriptase (AMV RT), 0.1 U/µl of Tfl DNA polymerase, 4 µl of total RNA template (levels in the range of 0.5-1 µg) and 27 µl of nuclease-free water. The reaction was incubated at 48 °C for 45 min, 94 °C for 2 min, followed by 40 cycles of 94 °C for 30 sec, 65 °C for 45 sec, 70 °C for 1 min, and a final extension step of 70 °C for 7 min. A control PCR assay in the absence of reverse transcriptase (RT) was performed in order to test for DNA contamination in the RNA
samples. A DNA-PCR assay was carried out under identical conditions with omission of RT and the 45 min incubation at 48 °C. A 984 bp fragment was amplified from the DNA sample from the PCV2 stock while no products were amplified from the PCV1 stock (Fig. 2). There was no evidence of amplified products in the RNA-enriched samples treated with DNase from either PCV1 or PCV2 stock (Fig. 2). These findings indicate that the RT-PCR assay can distinguish between PCV1 and PCV2 and that viral DNA, but not spliced Cap mRNA, is present in the PCV2 virus stock.

To detect PCV2 replication products, PCV free PK-15 cells (Fenaux et al., 2002) were infected with PCV1, PCV2 or UV-inactivated PCV2 at a multiplicity of infection of 0.1 for 1 hour followed by washing five times with PBS. Cells were gently scraped and collected at various times post-infection. Mock-infected PK-15 cells were collected at each time point and used as negative controls. Each sample was treated with RNALater™ (Ambion, Austin, TX) and kept at 4 °C for RNA extraction. Total RNA was isolated, treated with DNase, and amplified by RT-PCR assay as described above. The 594 bp fragment corresponding to the spliced Cap mRNA was detected as early as 14 hours post-infection (HPI) and was present through 48 HPI (Fig. 3). No products were amplified from RNA extracted from cells infected with PCV1, UV-inactivated PCV2 (Fig. 3) or from mock-infected PK-15 cells (data not shown). These data indicate that the spliced Cap mRNA is present only during PCV2 replication and confirms the specificity of this assay for PCV2. When RNA samples were not treated with DNase and amplified by the RT-PCR assay described above, a 984 bp fragment corresponding to PCV2 DNA was amplified with or without RT (Fig. 4). The 984 bp fragment was preferentially amplified by RT-PCR from the DNase-untreated RNA extracted from infected PK-15 cells at 24 HPI, even though the presence of spliced Cap mRNA could
be detected in the DNase-treated samples (Fig. 4). Therefore, DNase treatment was necessary for removal of DNA contamination in the RNA samples obtained from infected cells to detect the spliced Cap mRNA.

To confirm the identity of the PCR and RT-PCR products, the 984 and 594 bp amplification products were purified using the Qiaquick spin extraction kit (Qiagen), amplified, and sequenced at the Iowa State University Sequencing and Synthesis Facility using the primers described above. Analysis of the sequencing results revealed that the amplicons of both 985 and 594 bp fragments were PCV2-specific.

To evaluate the sensitivity of the RT-PCR assay, semi-confluent PK 15 cells were inoculated for 1 hour at 37 °C with the PCV2 stock (10^4 TCID₅₀/ml). The cells were washed five times with PBS buffer, fresh MEM medium (5% bovine calf serum and 1x antibiotics) was added, and the cells were incubated at 37 °C with 5% CO₂. The PK-15 cells were detached by gentle scraping at 48 HPI, disrupted manually, counted and 1×10^5 PCV2-infected cells were diluted with PBS buffer in 10-fold dilutions. Uninfected PK-15 cells were added to each dilution until the total numbers of cells was equal to 1×10^5. Each sample was treated with RNAlater™ (Ambion, Austin, TX) and the total RNA was extracted. The RT-PCR assay was performed on the 10^4 to 10⁻¹ cell dilutions in a 50 μl volume using the protocol described above. The spliced Cap mRNA was amplified from infected cells diluted to 10¹. These results suggest that the detection limit of the RT-PCR assay could be as few as 10 infected PK-15 cells based on the serial dilutions (Fig. 5). A previous study showed that without glucosamine treatment, only about 2% PCV-infected cells could be detected using indirect immunofluorescence assay (IFA) (Tischer et al., 1987). Compared to the detection ability of IFA, the RT-PCR assay described here appears highly sensitive.
We have demonstrated that our assay is able to detect the spliced Cap mRNA during virus replication. The assay is able to distinguish between PCV1 and PCV2 as no products were amplified from either PCV1 stock or PCV1-infected PK-15 cells. The ORF2 region was chosen as the target for this assay due to the variability in sequence between the two types of PCV. To distinguish between RNA products and viral DNA, the Cap primers were designed to amplify across the splice junction of the target sequence. The results also showed that the PCV2 DNA was easily detected from RNA extraction samples in the absence of DNase, indicating the necessity of DNase treatment in the RNA samples from infected cells. The single-tube RT-PCR protocol used in this study eliminates the need to open the tube to add PCR mix to the RT mix, thereby avoiding potential cross-contamination of samples. Although other researchers have reported the detection of virus replication using virus titration, ISH or Southern blot assays, these assays have either a low sensitivity or are time consuming. Our assay offers a rapid, specific and sensitive technique for detecting type-specific PCV2 while discriminating between replicating PCV2 and PCV2 virions. The incidence of PCV2-associated disease in pigs continues to increase globally, making identification of the cellular sites of replication important for understanding the pathogenesis of PCV2 infection and associated diseases. Our RT-PCR assay will assist in identifying the specific cell populations that support PCV2 replication in vivo and in vitro.

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References


Fig. 1. Strategy for design of oligonucleotide primers to detect 594 bp spliced Cap mRNA and 984 bp genomic DNA of PCV2. Arrows indicate the primers used in amplification of two different sized products, a 984 bp product amplified from virus DNA and a 594 bp product amplified from spliced Cap mRNA.

Fig. 2. Amplification of PCV2 DNA, but not mRNA, from virus stock. Arrows indicate locations of the 984 bp virus DNA amplification product. Products were separated on 1.5% agarose gels and visualized by UV light after staining for 10 min in ethidium bromide. M: marker (Gibco BRL 1kb plus DNA ladder, Life Technologies), +: presence of reverse transcriptase (RT) /with DNase treatment, -: absence of RT /without DNase treatment.
Fig. 3. Kinetics of PCV2 spliced Cap mRNA synthesis in infected PK-15 cells. Total cell RNA isolated at different time PI, indicated at the top of each lane. The single-tube RT-PCR was carried out with DNase-treated samples from PCV1 infected PK-15 cells at 48 HPI, UV-inactivated PCV2 infected cells at 48 HPI, and PCV2 infected cells at 1, 12, 14, 18, 24, and 48 HPI. Agarose gel electrophoresis of RT-PCR products demonstrate band size of 594 bp for spliced Cap mRNA. M: marker (Gibco BRL 1kb plus DNA ladder, Life Technologies), +: presence of RT, -: absence of RT.
Fig. 4. Amplification products from total RNA extracted from infected PK-15 cells with and without DNase treatment. The single-tube RT-PCR was carried out with DNase-treated and untreated samples from PCV2 infected cells at 1, 12 and 24 HPI. Agarose gel electrophoresis of RT-PCR products demonstrates band size of 984 bp for PCV2 genomic DNA. M: marker (Gibco BRL 1kb plus DNA ladder, Life Technologies), +: presence of RT /with DNase treatment, -: absence of RT /without DNase treatment.

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- 984 bp
- 594 bp
- 1000 bp
- 650 bp
Fig. 5. Sensitivity of RT-PCR on DNase-treated total RNA extractions from 10-fold dilution of $1 \times 10^4$ PCV2 infected PK-15 cells. Arrows indicate the location of the 594bp spliced Cap mRNA amplification product. M: marker (Gibco BRL 1kb plus DNA ladder, Life Technologies) and $10^4$, $10^3$, $10^2$, $10^1$, $10^0$, $10^{-1}$ PCV2-infected PK-15 cells, C: mock-infected PK-15 cells.
CHAPTER 3. DETECTION OF PCV2 CAPSID TRANSCRIPT IN PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMCS) INFECTED IN VITRO

A paper to be submitted to Veterinary Microbiology

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Abstract

The presence of PCV2 DNA and/or spliced capsid mRNA (Cap mRNA), as a measure of replication, was assessed, following addition of PCV2 to resting or concanavalin A (ConA) stimulated peripheral blood mononuclear cells (PBMCs) using real-time PCR or real-time RT-PCR assays, respectively. The results of the study demonstrated that PCV2 replication increased in infected PBMCs over time. Replication within infected PBMCs was significantly \(P<0.05\) increased when PBMCs were stimulated with ConA, compared to unstimulated PBMCs. The data showed a strong correlation between the level of PCV2 Cap mRNA and the level of viral DNA in the ConA stimulated PBMCs. Moreover, PCV2 replication was also assessed in T lymphocyte- and monocyte-enriched or monocyte-depleted PBMCs populations which were stimulated with ConA for 3 days. It was demonstrated that
the enriched T lymphocytes and the monocyte-depleted PBMCs had significantly higher Cap mRNA and viral DNA levels ($P<0.05$) compared to the monocyte-enriched population, indicating that PCV2 replicates in lymphocytes, particularly T lymphocytes, in addition to monocytes following stimulation. These results suggest that the presence of activated T lymphocytes may play an important role in PCV2 replication and the development of clinical disease.

**Keywords:** Peripheral blood mononuclear cells; PCV2; Real-time RT-PCR

**Introduction**

Porcine circovirus (PCV) is a circular single-stranded DNA virus in the *Circoviridae* family (Tischer *et al*., 1982; Buhk *et al*., 1985; Meehan *et al*., 1997). Currently, two PCV viruses are recognized, porcine circovirus type 2 (PCV2) and PCV type 1 (PCV1). PCV1 was first isolated from a porcine kidney cell line (PK-15) (ATCC CCL-33) (Tischer *et al*., 1974) while PCV2 was first reported to be associated with pigs exhibiting the post-weaning multisystemic wasting syndrome (PMWS) (Clark, 1997). The depletion of lymphocytes in the lymphoid follicles and their replacement by macrophages in conjunction with the presence of PCV2 antigen is a consistent finding associated with PMWS (Allan *et al*., 1999; Meehan *et al*., 1998). Nucleotide sequence analysis of PCV2 associated with PMWS revealed differences when compared to PCV1. While PCV1 is considered non-pathogenic, PCV2 is considered to be pathogenic and has been associated with a number of pathological disease syndromes including PMWS, enteritis, pneumonia, abortion, porcine respiratory disease complex (PRDC) and porcine dermatitis and nephropathy syndrome (PDNS) (Allan and Ellis, 2000; Segales *et al*., 2004).
Previous studies demonstrated that PCV has an ambisense circular genome (Tischer et al., 1982) that encodes proteins by the encapsidated viral DNA and by the complementary DNA of the replication intermediate synthesized in the host. The encapsidated viral DNA codes for the replication associated proteins and non-structural proteins, while the complementary strand synthesized during replication codes for the capsid protein (Cap) (Cheung, 2003a, b; Cheung and Bolin, 2002; Mankertz and Hillenbrand, 2001, 2002; Mankertz et al., 1998a; Mankertz et al., 1998b; Nawagitgul et al., 2000). For Cap expression, it is believed that the complementary strand is synthesized as a consequence of creating an intermediate double-stranded replication form (RF) (Meerts et al., 2005). A previous study showed that during productive PCV2 infection of PK-15 cells, a viral capsid mRNA, five replication-associated mRNAs, and three non-structural-associated mRNAs are generated and the capsid mRNA is a spliced RNA (Cheung, 2003b).

*In situ* hybridization (ISH) and polymerase chain reaction (PCR) assays have been used to detect PCV2 DNA in multiple organs and cell types recovered from infected pigs, however, these assays fail to differentiate between replicating virus and virion DNA. Recently, we developed a single-tube reverse transcription-PCR (RT-PCR) assay capable of detecting PCV2 spliced capsid mRNA (Cap mRNA), enabling us to discriminate between replicating PCV2 and PCV2 virions in PK-15 cells (Yu et al., 2005). However, this assay did not allow quantification of the viral product. To overcome this problem, a quantitative real-time RT-PCR assay to detect PCV2 Cap mRNA was developed and assessed in this study.

It has been shown that the viremia caused by PCV2 is both cell-free and cell-associated, but the cell-associated form appears to be more apparent and long-lasting (Pensaert et al., 2004). However, it is unknown whether the PCV2 found in association with
PBMCs resulted from active replication within the different cell populations, or was due to non-replicating virus that had either been adsorbed to or endocytosed by the cells. It has been demonstrated that increased PCV2 genome levels in serum or plasma samples recovered from infected pigs with clinical disease have been observed when compared to infected healthy controls (Brunborg et al., 2004; Liu et al., 2000). However, there was no report about increased PCV2 genome levels in PBMCs from PMWS affected pigs. In addition, several experimental studies have reported that immunostimulation of pigs results in an increased PCV2 replication and an increased incidence of PMWS (Krakowka et al., 2001; Kyriakis et al., 2002). To address whether immune stimulation may result in increased PCV2 replication in PBMCs, the study reported here assessed PCV2 replication in cultured PBMCs with or without mitogen stimulation at different time points by detecting PCV2 DNA and Cap mRNA using a real-time PCR and real-time RT-PCR assays, respectively. To further identify the specific PBMC populations in which PCV2 replicates, PCV2 infected and concanavalin A (ConA) stimulated PBMCs were sorted by flow cytometry into CD3⁺ (T lymphocyte), SWC3⁺ (monocyte), and SWC3⁻ (PBMCs with monocytes removed) populations. Propidium iodide (PI) was used in the flow cytometric sorting to make sure each collected cell population was viable cells. The various populations were evaluated for PCV2 DNA and Cap mRNA levels to ascertain cell populations permissive for PCV2 replication.

Materials and methods

Virus

The strain of PCV2 used in this study was PCV2 isolate 40895 (GenBank Accession No. AF264042) that has been shown to induce PMWS in pigs and has been adapted to grow in PK-15 cells. The titer of the in vitro propagated PCV2 was $1 \times 10^4$ 50% tissue culture
infective dose (TCID$_{50}$)/ml as determined by an immunofluorescence assay (IFA) (Fenaux et al., 2002). All PK-15 cells used in this study had been confirmed to be PCV-free (Fenaux et al., 2002).

**Experimental design**

PBMCs were isolated by density centrifugation using FicollHypaque (Histopaque 1077; Sigma, St Louis, MO) from four PCV2-free crossbred pigs using standard methods (Waters et al., 2000) and resuspended in complete medium (RPMI 1640 supplemented with 10% fetal calf serum, 0.05 mM 2-mercaptoethanol, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 1% non-essential amino acids 100x (GibcoBRL, Life Technologies, Carlsbad, CA), and 2 mM L-glutamine (Sigma, St. louis, MO)). The isolated PBMCs ($1 \times 10^6$ cells/sample) were infected with PCV2 at a multiplicity of infection (MOI) of approximately 0.1 or sham infected with media for one hour followed by washing 3 times with phosphate-buffered saline (PBS) and resuspension in complete medium. The PCV2 infected and non-infected PBMCs were incubated at 37 °C with 5% CO$_2$ with or without the mitogen, ConA at 5 μg/ml (Sigma, St. Louis, MO). Cells were harvested at 1, 18, 38, or 72 hours post-infection (HPI). At each time point, the cells were washed four times with PBS to remove free PCV2. Total cells were enumerated after the addition of a non-enzymatic cell dissociation solution (Sigma-Aldrich, St. Louis, MO) and cell viability was determined using trypan blue exclusion. An aliquot containing $1 \times 10^5$ viable cells per sample was centrifuged and the cell pellet was suspended in 200 μl RNAlater (Ambion, Austin, TX) and used for total RNA isolation. An additional aliquot of $1 \times 10^5$ viable cells was placed in 200 μl PBS and used for total DNA isolation. This experiment was performed twice.
Enrichment of cellular subpopulations from PCV2 infected PBMCs

PBMCs with and without PCV2 infection as described above were incubated 3 days with and without ConA (5 μg/ml) at 37 °C with 5% CO₂. Cells were then labeled with FITC-conjugated mouse anti-CD3 antibodies (PPT3, Southern Biotech, Birmingham, AL) to identify T lymphocytes or FITC-conjugated mouse anti-porcine SWC3 antibodies (74-22-15, Southern Biotech, Birmingham, AL) to detect monocytes, followed by staining with propidium iodide (PI) to identify viable cells. The CD3⁺PI⁻, SWC3⁺PI⁻ and SWC3⁺PI⁺ (SWC3⁻ cells consist of PBMCs with monocytes removed) cell fractions were collected using an EPICS ALTRA flow cytometer (Beckman Coulter, Miami, FL) at the Iowa State University Cell facility. Each fraction was counted, and 1×10⁵ viable cells was suspended in 200 μl RNAlater for total RNA isolation with an additional 1×10⁵ viable cells placed in 200 μl PBS for DNA isolation.

Nucleic acid extraction

Total RNA was extracted using the Versagene total RNA purification kit (Gentra Systems, Minneapolis, MN) according to the manufacturer’s instruction, and solubilized in 70 μl of elution solution. The RNA extracts were stored at −80 °C until real-time RT-PCR amplification was carried out. Viral DNA was extracted from each cell sample using the QIAamp DNA Mini kit (QIAGEN, Valencia, CA) according to the manufacturer’s instruction and solubilized in 100 μl of elution buffer. The DNA extracts were stored at −20 °C until assayed.

Real-time RT-PCR assay for PCV2 Cap mRNA

The forward primer (5'-AGATGCCATTTTTCCTTC-3'), reverse primer (5'-GCTCCACATTCAATAACTATGAC-3') and the probe used in quantification of spliced
capsid transcript of PCV2 were designed with the Primer 3 software (Rozen and Skaletsky, 2000; http://www-genome.wi.mit.edu/cgi-bin/primer/primer3 www.cgi), based on sequence data of PCV2 isolate 40895 (GenBank Accession No. AF264042). The probe (5'-/56-FAM/TCTTCTTCTGGTAACGGCTC/3BHQ_1/-3') was labeled with a fluorescent reporter dye, 6-carboxyfluorescein (FAM) at 5’ end and a quencher dye, Black Hole Quencher 1 (BHQ_1) at 3’ end. To avoid amplification interference from potential DNA contamination, the reverse primer was designed to span the splice junction to ensure that PCV2 DNA was not amplified.

To make a RNA standard, a 594 bp RT-PCR product was amplified from Cap mRNA with the forward primer (5'-GGAGTCAAGAACAGGTTTGGGTG-3') and the reverse primer (5'-AGACTCCCGCTCTCCAACAAG-3'). The product was ligated directly into the pCRII cloning vector (TA Cloning kit, Invitrogen Corporation, Carlsbad, CA) according to the manufacturer’s instructions. After transformation into competent *E. coli* (Invitrogen Corporation, Carlsbad, CA), individual bacterial colonies were screened for recombinant plasmid using blue/white color selection. Purified, cloned plasmid DNA was prepared using a QIAPrep Spin miniprep kit (QIAGEN, Valencia, CA) and the sequence and orientation of the insert was verified by DNA sequencing at the DNA Sequencing and Synthesis Facility, Iowa State University. The plasmid was linearized using the NotI restriction enzyme and *in vitro* transcribed using the SP6 RNA polymerase of RiboMax large scale RNA production systems (Promega, Madison, WI) as described by the manufacturer. The RNA transcript was purified using a standard phenol-chloroform extraction method. The final RNA pellet was resuspended in DNase/RNase-free water. After determining the concentration by absorbance at 260 nm using NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies,
Wilmington, DE), the RNA transcript was diluted to a concentration of 10⁸ copies/µl in DNase/RNase-free water and stored at −80 °C until use.

The quantification of Cap mRNA was performed using real-time RT-PCR on a Rotor-Gene RG-300 (Corbett Research, Sydney, AU). The 20 µl reaction mixture consisted of 10 µl 2x TaqMan Universal PCR master mix buffer (Applied Biosystems, Foster City, CA); 0.1 µl SuperScript III reverse transcriptase (200 U/µl) (Invitrogen, Carlsbad, CA); 0.2 µl RNaseout (40 U/µl) (Invitrogen, Carlsbad, CA); 2 µl of forward primer with final concentration 450 nM and 2 µl of reverse primer with final concentration 150 nM; 0.8 µl of TaqMan probe with final concentration 150 nM; 2.9 µl DNase/RNase-free water and 2 µl of 10-fold dilutions of RNA standard or 2 µl of extracted total RNA from each sample. The amplification was performed at 55 °C for 45 min, 95 °C for 10 min; then for 45 cycles at 95 °C for 15 sec each and a single cycle at 60 °C for 60 sec. The fluorescence was read at the end of each round of amplification. All standard dilutions and unknown samples were run in duplicate. Standard curves were accepted when the coefficients of correlation (r²) were > 0.99. Quantification of PCV2 Cap mRNA were achieved by comparing the threshold cycle (C₉) value of the input sample RNA with the C₉ value of the standard RNA.

The sensitivity of the Cap mRNA detection assay was evaluated using 10-fold serial dilutions of the RNA standard described above. To ensure RNA specificity, the described real-time RT-PCR procedures were performed with and without reverse transcriptase as well as performed on total RNA samples isolated 48 hours after infecting PK-15 cells with PCV1 (Tischer et al., 1974). Independent assays were performed at least twice. To evaluate intra-assay variability and the inter-assay reproducibility, 10-fold serial dilutions of the Cap mRNA standard were tested in triplicate in three separate experiments.
**Real-time PCR assay for PCV2 DNA**

The PCV2 real-time PCR assay was performed using the Rotor-Gene RG-300 (Corbett Research, Sydney, AU) as previously described (Opriessnig et al., 2003) with some modifications. Briefly, the probe (5'-/56-FAM/CCAGCAATCAGACCCCGTTGGAATG/3′-BHQ_1 A3') was labeled with a fluorescent reporter dye, FAM at 5′ end and a quencher dye, BHQ_1 at 3′ end. The PCR reaction was performed in a 25 μl volume containing: 12.5 μl TaqMan Universal PCR master mix (Applied Biosystems, Foster City, CA); 5 μl of each forward and reverse primer (final concentration 450 nM); 1 μl of TaqMan probe (final concentration 150 nM); 1.5 μl of 10-fold dilutions of PCV2 plasmid DNA or DNA extracts. Quantification of viral DNA was achieved by comparing the CT value of the input sample DNA with the CT value of the standard template DNA. All dilutions of PCV2 plasmid DNA and unknown samples were tested in duplicate.

**Statistical analysis**

Analysis of variance (ANOVA) was used to analyze the data. P values less than 0.05 were considered to be statistically significant for all test procedures. The correlation of the copy numbers of PCV2 Cap mRNA and viral DNA was evaluated by nonparametric correlations test, Spearman’s Rho in Multivariate Methods using JMP5.1 (SAS Institute Inc., Cary, NC).

**Results**

**Analytical performance of the real-time RT-PCR**

A 151 bp fragment was amplified in the real-time RT-PCR assay that corresponded with the anticipated size of Cap mRNA. Standard curves ranging from 500 to $5 \times 10^7$
copies/reaction were constructed using serial dilutions of a RNA standard. The results were linear over a 5-log range demonstrating the dynamic range of the assay. A typical curve generated from the analysis of 10-fold dilutions of RNA standard is shown in Fig. 1. A strong linear relationship ($R^2 > 0.99$) between the $C_T$ values and the $\log_{10}$ of the input copy number was observed. The coefficient of variation (%) of the mean $C_T$ values within the run was in the range of 0.4-1.4% and from run to run was in the range of 1.0-2.0%. In the absence of reverse transcriptase, the Cap mRNA can not be detected. No RT-PCR product was detected from RNA samples recovered from PCV1 infected PK-15 cells.

**Measurement of PCV2 Cap mRNA and viral DNA production in cultured PBMCs**

To detect PCV2 replication in PBMCs at different time post-infection *in vitro*, the amount of Cap mRNA and viral DNA were measured. As expected, PCV2 DNA was detected as early as 1 HPI, whereas no Cap mRNA was detected at that time. The Cap mRNA was detected in the cultured PBMCs beginning at 18 HPI. Following infection with PCV2, Cap mRNA copy numbers increased in both ConA-stimulated and non-stimulated PBMCs over time; however, the levels of Cap mRNA were significantly higher ($P<0.05$) in ConA stimulated PBMCs compared to non-stimulated PBMCs at all three time points post-infection (Table 1). The PCV2 DNA copy number also increased in infected PBMCs over time, and as with the Cap mRNA copy numbers, the levels were significantly higher ($P<0.05$) in ConA-stimulated PBMCs compared to non-stimulated PBMCs at 18, 38, and 72 HPI (Table 1). A strong correlation existed between the levels of Cap mRNA and viral DNA in ConA stimulated PBMCs ($r = 0.97, P<0.001$) whereas no significant correlation was observed in non-stimulated PBMCs. Neither the Cap mRNA nor viral DNA product was amplified from sham-infected PBMCs at any time point.
Measurement of PCV2 Cap mRNA and viral DNA production in PBMC subpopulations

To detect the specific cell population supporting PCV2 replication during the active viral replication period, T lymphocytes, monocytes and monocyte-depleted PBMCs were sorted from ConA stimulated PBMCs three days post-infection with PCV2. The purity of monocyte (SWC3⁺) and T lymphocyte (CD3⁺) populations were >90%. Less than 1% SWC3⁺ cells were detected in the SWC3⁻ cell population which consisted primarily of lymphocytes (data not shown). The T lymphocyte population (CD3⁺) had increased levels of Cap mRNA consistent with PCV2 replication (Fig. 2). The PCV2 Cap mRNA and viral DNA levels were significantly lower in the SWC3⁺ monocyte-enriched population than all other cell populations (P<0.05). The level of PCV2 replication in the SWC3⁻ group was equivalent to the CD3⁺ and total PBMC populations, and is greater than SWC3⁺ cells.

Discussion

A previous study demonstrated PCV2 was associated with PBMCs from PCV2 infected pigs (Pensaert et al., 2004). However, no detailed assessment of PCV2 replication within porcine PBMCs has been performed. Therefore, it was unclear whether PBMCs are capable of supporting PCV2 replication or if the virus had been adsorbed to or endocytosed by the cells. To date, the population of cells associated with PCV2 replication has not been well characterized. Microscopic lesions associated with PCV2-induced disease have been characterized by lymphoid depletion (Allan and Ellis, 2000). However, the pathogenic mechanism by which PCV2 induces lymphoid depletion and the role virus infection of lymphocytes plays in the disease process has not been fully elucidated. Previous investigators have suggested that immune stimulation increases the level of PCV2 detected in the blood
and tissues and it was hypothesized that this increase in viral load may be related to the increased pathological changes associated with PMWS (Krakowka et al., 2001; Kyriakis et al., 2002; Opriessnig et al., 2003). In order to investigate PCV2 replication in PBMCs as well as to define the temporal relationship between cellular activation (e.g., ConA stimulation) and PCV2 replication, a quantitative assay to measure replication-associated PCV2 Cap mRNA was utilized (Yu et al., 2005). In order to demonstrate that active viral replication would result in increased numbers of PCV2 virions, a real-time PCR assay was used to measure PCV2 DNA levels. The results showed that both the PCV2 Cap mRNA copy number and the PCV2 DNA copy number increased in PBMCs in vitro and significantly increased (P<0.05) in PBMCs in association with ConA stimulation. These results support the hypothesis that activation of lymphocytes (i.e., immune stimulation) enhances PCV2 replication (Krakowka et al., 2001; Kyriakis et al., 2002; Opriessnig et al., 2003).

In order to further identify the specific cell populations in which PCV2 actively replicates, infected PBMCs were stimulated with ConA for 3 days, and then sorted by flow cytometry into viable T lymphocytes (CD3^+), monocytic/macrophage cells (SWC3^+), and PBMCs with monocytic/macrophage cells removed (SWC3^+). Both the real-time RT-PCR assay and real-time PCR assay were used to quantify the replication of PCV2 in these cell populations. Based upon the results of both assays it was determined that the number of PCV2 Cap mRNA and PCV2 virions in SWC3^+ monocyte-depleted PBMCs or CD3^+ T lymphocytes were significantly increased (P < 0.05) compared to the levels in the monocytic/macrophage cells (SWC3^+). This suggests that lymphocytes are a primary site for PCV2 replication when they are activated. Previous studies using ISH or IHC staining demonstrated that PCV2 nucleic acid or antigen in PMWS-affected pigs is primarily
localized to the cytoplasm of histiocytes, multinucleate giant cells, and other monocyte/macrophage lineage cells (Allan and Ellis, 2000; Chianini et al., 2003; Ellis et al., 1998; Gilpin et al., 2003; Rosell et al., 1999). However, several in vitro studies have failed to observe PCV2 replication in monocyte/macrophage lineage cells (Gilpin et al., 2003; Meerts et al., 2005; Vincent et al., 2003). These studies suggested that monocytic cells may not represent the primary target for PCV2 replication, but that they acquired viral antigens or nucleic acid through phagocytosis (Gilpin et al., 2003). In vivo infected lymphocytes may be phagocytized immediately by macrophages present within the lymphoid tissues. Thus, the accumulated PCV2 in macrophage was detected by ISH or IHC assays which may not be sensitive enough to detect lower amounts of virus in lymphocytes in vivo. In support of this hypothesis, it has been demonstrated that more than $10^7$ PCV2 genome copies per 500 ng of tissue has been obtained from mesenteric lymph nodes of pigs with PMWS (Brunborg et al., 2004). However, the estimated minimal viral load of $10^8$ PCV2 genome copies per 500 ng DNA is required in order to detect viral antigen using IHC assay (Brunborg et al., 2004). In our study, we were able to detect both the PCV2 Cap mRNA and viral DNA in the SWC3$^+$ monocytic cell population. However, we cultured these cells with lymphocytes for 3 days prior to separation. It is possible that some of the Cap mRNA in these cells may have originated from lymphocytes subsequently taken up by these monocytic cells. Whether the detection of PCV2 Cap mRNA and viral DNA in SWC3$^+$ cells was due to uptake of infected lymphocytes or due to active replication of PCV2 within them was not investigated, so a low level of replication in monocytic cells can not be excluded. However, the fact that the level of viral Cap mRNA was about 100-fold lower ($P < 0.05$) in the SWC3$^+$ cells suggested that these cells are not the primary target for PCV2 replication.
In contrast to a previous study (Gilpin et al., 2003), our study demonstrated that both viral RNA and DNA levels were increased in T lymphocyte populations compared to the SWC3⁺ monocyte population. These results confirm that PCV2 is lymphotropic as has been observed with other Circoviridae family members such as chicken anemia virus and TT virus (Adair, 2000; Maggi et al., 2001). One significant difference between the study reported here and that of Gilpin et al. (Gilpin et al., 2003) is that the cells used in this study were stimulated with ConA in addition to being infected with PCV2. We stimulated the cells to increase viable cells as typically more than 80% of the lymphocytes in culture die within the first 3 days in the absence of stimuli. Thus, total cellular viability was enhanced by ConA stimulation and this allowed for increased viral replication and the ability to detect replication within the lymphocyte population. In addition, immune stimulation has been associated with increased viral loads and clinical disease (i.e., PMWS) in pigs (Krakowka et al., 2001; Kyriakis et al., 2002; Opriessnig et al., 2003). However, by stimulating PBMCs with ConA, selection for specific lymphocyte subpopulations may have occurred. Subsequent studies will be required to further define the subpopulation(s) of lymphocytes in which PCV2 replicates.

Due to the lack of reagents to identify and stimulate porcine B cells, the PCV2 replication in this lymphocyte population was not investigated. However, the copy number of PCV2 Cap mRNA and viral DNA in PBMCs depleted of monocytes (SWC3⁻) tended to be higher than the levels in either the complete PBMCs or the T lymphocyte-enriched populations, suggesting that B cells may also allow viral replication.

Previously, we have developed a single-tube RT-PCR assay that detects PCV2 Cap mRNA and is able to discriminate between replicating PCV2 and PCV2 virions in PK-15
cells (Yu et al., 2005). However, this assay was not quantitative. In the present study, a real-time RT-PCR assay to detect and quantify PCV2 Cap mRNA was developed. The primers and the probe were designed to detect only Cap mRNA transcript. No PCV2 DNA was amplified. The evaluation of the performance of this real-time RT-PCR assay demonstrated that this assay was a sensitive, specific, and highly reproducible technique for assessing PCV2 replication. Moreover, this real-time RT-PCR method is performed in a closed tube and requires no post-PCR analyses. Therefore, outside contamination by viral DNA was eliminated.

The results of this study demonstrated that PBMCs support PCV2 replication and that in vitro stimulation of PBMCs with ConA enhanced viral replication. Using flow cytometric sorting of PBMCs, PCV2 replication in T lymphocytes was also demonstrated. While a low level (i.e., 100-fold less) of viral replication was detected in the monocytic/macrophage cells (SWC3+), it was not determined whether this represented viral replication or phagocytic uptake of viral products. Further studies to identify the specific subpopulations of cells permitting PCV2 replication in vivo are required to better understand the pathogenesis of PMWS and the mechanisms by which PCV2 infection progresses to PMWS.

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References


Fig. 1. A standard curve derived from the amplification of a dilution series containing $5 \times 10^2$ to $5 \times 10^7$ copies/reaction of the RNA standard for PCV2 spliced capsid mRNA. The standard curve was plotted for starting copy number versus threshold cycle ($C_T$) and had a correlation coefficient ($R^2$) of 0.997.
Fig. 2. Quantification of PCV2 spliced capsid mRNA (A) and PCV2 DNA (B) in infected peripheral blood mononuclear cell populations with concanavalin A stimulation for 3 days. The populations include peripheral blood mononuclear cell (PBMCs) without monocytes (Mon Neg), PBMCs (PBMC), T lymphocytes (CD3) and monocytes (Mon Pos).

* Significant difference at $P<0.05$ within sorted cell populations. Error bars represent standard deviation from the average values.
Table 1. *In vitro* kinetics of PCV2 replication in resting or concanavalin A (ConA)-stimulated peripheral blood mononuclear cells (PBMCs).

<table>
<thead>
<tr>
<th>HPI</th>
<th>PCV2 spliced capsid mRNA copy number(^1) (Mean ± Std Dev)</th>
<th>PCV2 DNA copy number (Mean ± Std Dev)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-stimulated</td>
<td>ConA-stimulated</td>
</tr>
<tr>
<td>1</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>18</td>
<td>4.58 ± 0.26</td>
<td>5.45 ± 0.17*</td>
</tr>
<tr>
<td>38</td>
<td>4.64 ± 1.14</td>
<td>7.43 ± 0.07*</td>
</tr>
<tr>
<td>72</td>
<td>4.78 ± 1.60</td>
<td>8.69 ± 0.09*</td>
</tr>
</tbody>
</table>

\(^1\)Copy number reported as the log\(_{10}\) at each time point.

\(^2\)PBMC cultures were stimulated with 5 μg/mL of ConA at the same time when PCV2 was added to the cells.

Viral replication was evaluated by measuring the level of PCV2 spliced capsid mRNA and viral DNA using real-time reverse transcription (RT)-PCR and a real-time PCR assay, respectively.

ND = Not Detected

*Significant difference between PCV2 infected PBMCs that were ConA stimulated in comparison to non-stimulated PBMCs (\(P<0.05\)).
CHAPTER 4. PORCINE CIRCOVIRUS TYPE 2 (PCV2)
DISTRIBUTION AND REPLICATION IN TISSUES AND IMMUNE
CELLS IN EARLY INFECTED PIGS

A paper to be submitted to Viral Immunology and Immunopathology

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Abstract

Replication of porcine circovirus type 2 (PCV2) in pigs, as measured by spliced capsid mRNA (Cap mRNA) and viral DNA, was investigated following experimental infection. Peripheral blood mononuclear cells (PBMCs), and tissues from the bronchial lymph nodes (BLN), inguinal lymph nodes (ILN), tonsils, lungs, liver, kidneys, spleen and thymus from infected pigs on different days post-infection (DPI) were assessed. PCV2 replication differed dramatically between tissues from the same infected pig. The virus actively replicated in most tested tissues at 14 DPI in association with severe PCV2 associated lesions and abundant PCV2 antigen. The PCV2 Cap mRNA was only detected at 13 DPI in PBMCs from infected pigs, suggesting replication of the virus in circulating blood and peripheral blood is transient and thus not a major site for PCV2 replication \textit{in vivo}.

Evaluation of the Cap mRNA and viral DNA synthesis in T and B lymphocyte and monocyte populations from PBMCs and BLN at various intervals post-inoculation revealed the
replication of PCV2 in all cell subpopulations; viral replication in B lymphocytes was greater than in monocytes from BLN at 14 DPI indicating that B lymphocytes are permissive for PCV2 replication. These findings further our understanding of the cell types permissive for PCV2 replication and the pathogenesis of PCV2 infection in vivo.

**Keywords:** PCV2; Real-time RT-PCR; *In vivo*

### Introduction

Porcine circovirus type 2 (PCV2) is a circular single-stranded DNA virus in the Circoviridae family (Tischer et al., 1982; Buhk et al., 1985; Meehan et al., 1997). It was first associated with pigs exhibiting the postweaning multisystemic wasting syndrome (PMWS) in Western Canada (Clark, 1997). Depletion of lymphocytes in the lymphoid follicles and their replacement by macrophages make up the hallmark lesions observed in this syndrome (Allan et al., 1999; Meehan et al., 1998). Infection with PCV2 has also been associated with a number of other pathologic disease syndromes including enteritis, pneumonia and respiratory disease, abortion and porcine dermatitis and nephropathy syndrome (PDNS) (Allan and Ellis, 2000; Segales et al., 2004b).

*In situ* hybridization (ISH) and polymerase chain reaction (PCR) assays have been used to detect PCV2 DNA in tissues and cells from infected pigs. In addition, the lymphocyte depletion of follicular and interfollicular lymphoid tissues together with the macrophage infiltration of lymphoid tissues observed in PMWS affected pigs are highly correlated with a decrease in numbers of circulating B and T cells (Chianini et al., 2003; Clark, 1997; Darwich et al., 2002; Nielsen et al., 2003; Quintana et al., 2001; Rosell et al., 1999; Sarli et al., 2001; Segales et al., 2000; Shibahara et al., 2000). However, it is not clear which tissue or cell type is the primary site for PCV2 infection and replication. Recently we demonstrated
that PCV2 replicates in PBMCs using a quantitative real-time reverse transcription-PCR (RT-PCR) assay that detects PCV2 spliced capsid mRNA (Cap mRNA) and allowing discrimination between replicating PCV2 and PCV2 virions (Yu et al., 2004). The aim of this study was to understand the replication of PCV2 in vivo and identify cell populations that are permissive for PCV2 replication in vivo. In this study, we investigated the levels of PCV2 Cap mRNA and viral DNA in PBMCs, bronchoalveolar lavage (BAL) cells, and multiple tissues (bronchial lymph nodes (BLN), inguinal lymph nodes (ILN), lung, tonsil, thymus, spleen, liver and kidney) from pigs during the acute phase PCV2 infection as well as the viral DNA levels in serum and cell-free BAL fluid. The microscopic lesions and the amount of PCV2 antigen in tissues was measured using histopathology and immunohistochemistry (IHC), respectively. PCV2 antibody levels in serum and BAL fluid were also assessed. Specific immune cell populations were sorted by AutoMACS from peripheral blood or BLN mononuclear cells and the PCV2 infection and replication in each cell population was further measured by real-time RT-PCR and real-time PCR assays, respectively. Our studies show that PCV2 replication is dramatically different between tissues from the same pig and suggest that lymphocytes are permissive for PCV2 replication while monocytes may be the site for PCV2 persistence in the infected pigs.

Materials and methods

PCV2 inocula

The challenge inoculum was prepared from a PCV2 virus stock derived from the molecular clone of PCV2 isolate 40895 (GenBank Accession No. AF264042). This virus was isolated from the spleen of a pig with naturally occurring PMWS (Fenaux et al., 2002). The titer of the PCV2 inoculum was $1 \times 10^{4.75}$ 50% tissue culture infective dose (TCID$_{50}$/ml as
determined by titration on PCV-free PK-15 cells using a previously described immunofluorescence assay (IFA) (Fenaux et al., 2002).

**Experimental design**

Twenty-four PCV2-free cross-bred pigs were used in this study. The pigs were assigned to two rooms (twelve pigs/room) on the day of challenge (trial day 0). Twelve pigs in one room were inoculated intranasally at the age of 4 to 5 weeks with 5 ml of the PCV2 inoculum. The remaining pigs housed in a separate room were sham-inoculated with 5 ml of PCV-free PK15 cell lysate and served as negative controls. All study procedures and animal care were conducted in accordance within the guidelines and under the approval of the Iowa State University Institutional Committee on Animal Care and Use.

**Necropsy and sample collection**

Three pigs from each group were necropsied at 3, 7, 14 and 21 days post-infection (DPI). Tissue samples from BLN, ILN, lungs, tonsils, thymus, spleen, liver and kidney of each pig were collected at necropsy and fixed in 10% formalin for histological examination and IHC assay. Fresh BLN samples were also placed immediately in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin for isolation of mononuclear cells. In addition, pieces of each tissue described above were snap frozen on dry ice and stored at −80 °C until used for isolation of total RNA or DNA.

The right lung of each necropsied pig was lavaged with 50 ml of collecting solution (sterile phosphate-buffered saline (PBS) with 1% bovine serum albumin (BSA), 300 U/ml penicillin and 300 mg/ml streptomycin). The BAL was centrifuged (400 g, 10 min, 4 °C) to separate cells and cell-free lavage fluid. BAL cells (1x10^6) were resuspended in 200 μl RNALater (Ambion, Austin, TX) for total RNA and DNA isolation. The cell-free lavage fluid
was stored at -20 °C for PCV2 antibody detection and DNA isolation. Serum samples were collected at 0, 2, 6, 13, and 20 DPI and stored at -20 °C until used for DNA isolation or assayed for PCV2 antibodies. Concurrently, heparinized blood samples were collected for PBMCs isolation.

**Isolation of mononuclear cells from BLN and peripheral blood**

Fresh BLN tissues were cut into small pieces and pushed through a 70 μm pore-size nylon cell strainer (Falcon) to create single-cell suspensions. The cells were centrifuged at 300 g for 8-10 min and resuspended in 3 ml sterile water for 15 sec to lyse red blood cells immediately followed by the addition of 3.5% sodium chloride. Cells were washed with PBS twice and resuspended in complete medium (RPMI 1640 supplemented with 10% fetal calf serum, 0.05 mM 2-mercaptoethanol, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 1% non-essential amino acids 100x (GibcoBRL, Life Technologies, Carlsbad, CA), and 2 mM L-glutamine (Sigma, St. louis, MO)). PBMCs were isolated by Ficoll-hypaque (Histopaque 1077; Sigma, St Louis, MO) density centrifugation from collected peripheral blood using standard methods (Waters et al., 2000) and resuspended in complete medium.

**Enrichment of T, B lymphocytes and monocytes**

The isolated BLN mononuclear cells or the PBMCs were separated by positive selection using the AutoMACS, magnetic separation system (Miltenyi Biotec, Auburn CA), according to the manufacturer’s instructions. Briefly, the single-cell suspensions at a concentration of $3 \times 10^7$, $6 \times 10^7$, $6 \times 10^7$ cells were centrifuged followed by incubation with the following monoclonal antibodies; FITC-conjugated mouse anti-pig CD3 (PPT3, Southern Biotechnology Associates, Birmingham, AL) for T lymphocytes, FITC-conjugated mouse anti-pig CD21 (BB6-11C9.6, Southern Biotechnology Associates, Birmingham, AL) for B
lymphocytes and FITC-conjugated mouse anti-pig SWC3 (74-22-15, Southern Biotechnology Associates, Birmingham, AL) for monocytes/macrophages, respectively, for 10 min in the dark at 4-8 °C. The cells were washed twice with 2 ml cold MACS buffer (PBS containing 5 mM EDTA and 0.5% BSA) per 10\(^7\) cells and centrifuged at 300 g for 10 min. The supernatant was removed completely and the cells resuspended in 90 μl of MACS buffer per 10\(^7\) total cells. Ten μl of anti-FITC coated MicroBeads per 10\(^7\) total cells was then added and incubated for 15 min at 4-8 °C. The cells were again washed twice by adding 2 ml of buffer per 10\(^7\) cells and centrifuged at 300 g for 10 min followed by re-suspension in 500 μl of MACS buffer. The T, B lymphocyte and monocyte populations were separated using the autoMACS by positive selection. The purity of sorted cell fractions was assessed by flow cytometric analysis with a FACScan (Becton-Dickinson, Franklin Lakes, NJ). 7-amino-actinomycin D (7AAD) was added to each tested cell fractions before conduct the flow cytometric analysis to assess the cell viability. Each subpopulation was centrifuged and 1×10\(^6\) cells/ cell fraction was resuspended in 200 μl RNAlater (Ambion, Austin, TX ) for total RNA isolation and an additional 1×10\(^6\) cells placed in 200 μl PBS for DNA isolation.

**Extraction of nucleic acids**

To isolate the total RNA from collected tissues, small pieces of each frozen tissue described above was incubated at -20 °C for 16 hours in 2 ml of RNA later-ice frozen tissue transition solution (Ambion® Austin, TX). Twenty mg of each incubated tissue was homogenized and the total RNA was isolated using RNAqueous small scale phenol-free total RNA isolation kit (Ambion, Austin, TX) according to the manufacturer's instructions. To extract total RNA from a known number of each cell population including PBMCs, the purified T lymphocytes, B lymphocytes and monocytes, the Versagene RNA purification kit
(Gentra systems, Minneapolis, MN) was used according to the manufacturer’s instructions.

Genomic DNA was isolated from frozen tissues, serum, cell-free BAL fluid, and purified T
or B lymphocyte and monocyte preparations using the QIAamp DNA mini kit (Qiagen,
Valencia, CA) according to the manufacturer’s instructions. For DNA extraction of serum or
cell-free BAL fluid samples, 200 μl was used as starting material. For DNA extraction of
tissue samples, 10 mg spleen or 20 mg of each other tissue sample was used. For the
AutoMACs sorted immune cells, 1×10^6 cells/cell fraction were used. Each tissue sample was
added to lysis buffer with Proteinase K (20 mg/ml) and incubated at 55 °C for 6-7 hours until
completely lysed. The DNA was eluted in 100 μl elution buffer and storage at −20 °C until
use.

**Real-time RT-PCR assay and real-time PCR assay**

A real-time RT-PCR assay to detect the Cap mRNA of PCV2 was performed on total
RNA as previously described (Yu et al., 2004). The forward primer (5'-
AGATGCCATTTTTCCTTCTC-3'), reverse primer (5'-
GCTCCACATTCAATAACTATGAC-3') and the probe (5'/56-
FAM/TCTTCTTCTGCGTAACGCCTC/3BHQ_1/-3') were used to amplify and quantify
the Cap mRNA on Rotor-Gene RG-300 (Corbett Research, Sydney, AU). The probe was
labeled with a fluorescent reporter dye, 6-carboxyfluorescein (FAM) at 5’ end and a
quencher dye, Black Hole Quencher 1 (BHQ_1) at 3’ end. The reaction mixture was in a 20
μl final volume and the reactions were carried out at 55 °C for 45 min, 95 °C for 10 min, then
for 45 cycles at 95 °C for 15 sec and 60 °C for 60 sec. All unknown samples and standard
dilutions ranging from 500 to 5×10^6 copy numbers were run in duplicates. Quantification of
PCV2 Cap mRNA were achieved by comparing the threshold cycle \( (C_T) \) value of the input sample RNA with the \( C_T \) value of the standard RNA.

DNA extracts were used to quantify the amount of PCV2 genomic DNA by real-time PCR assay. The real-time PCR assay was performed using the Rotor-Gene RG-300 (Corbett Research, Sydney, AU) as previously described (Opriessnig et al., 2003) with some modifications. Briefly, the probe \( (5'\text{-/56}-\text{FAM/CCAGCAATCAGACCCCGTTGGAATG/3BHQ}_1/-3') \) was labeled with a fluorescent reporter dye, FAM at 5' end and a quencher dye, BHQ_1 at 3' end. The PCR reaction was carried out in a 25 µl final volume. All reactions were carried out in duplicate. In addition, each run included 10-fold dilutions ranging from 20 to \( 2\times10^5 \) copy numbers of PCV2 plasmid DNA to generate a standard curve. Quantification of viral DNA was achieved by comparing the \( C_T \) value of the input sample DNA with the \( C_T \) value of the standard template DNA.

**Histopathology and Immunohistochemistry**

Tissue samples of BLN, ILN, lung, tonsil, thymus, spleen, liver and kidney collected at each necropsy were fixed in 10% neutral buffered formalin, embedded in paraffin wax, and processed routinely for histological examination. Microscopic lesions were evaluated blindly by two veterinary pathologists (T. Opriessnig, P. G. Halbur) using a previously described scoring system (Opriessnig et al., 2004).

PCV2-specific antigen was measured by IHC on sections of paraffin embedded tissue samples using a rabbit polyclonal antiserum as previously described (Sorden et al., 1999). The amount and distribution of PCV2 antigen was assessed by evaluation of IHC signals of each tissue section ranging from 0 (= no signal) to 3 (= strong positive signals) as previously
described (Opriessnig et al., 2004) in a blinded fashion. The mean group score was
determined for each tissue and compared.

**PCV2 antibody detection**

A PCV2 enzyme-linked immunosorbent assay (ELISA) based on the recombinant
open reading frame 2 (ORF2) capsid protein of PCV2 was performed to detect antibodies to
PCV2 in serum samples as described previously (Nawagitgul et al., 2000). The cut-off values
used were as follows: samples with S/P ratio (sample-to-positive ratio) < 0.12 were
considered negative, ratios between 0.12 to 0.2 were considered suspect, and ratios of > 0.2
were considered positive (Nawagitgul et al., 2002). PCV2-specific antibodies were assessed
in BAL using an ELISA assay as described previously with some modification (Nawagitgul
et al., 2002). Briefly, Immulon-2HB 96-well plates (Dynex, Chantilly, VA) were coated with
100 μl of ORF2 protein and the negative control, wide type (WT) protein. Plates were
incubated at 4 °C for 40 hours and washed 3 times with 0.05% Tween 20 in PBS (PBS-T).
Each BAL sample (100 μl) was added to duplicate wells and incubated at 37 °C for 30 min,
washed 3 times with PBS-T, then incubated with peroxidase-labeled goat anti-swine IgG
(Kirkegaard and Perry, Gaithersburg, MD), peroxidase-labeled goat anti-swine IgA (Bethyl,
TX) or peroxidase-labeled goat anti-swine IgM (Bethyl, TX) at 37 °C for 30 min. The
substrate, 2,2'-azino-di-(3- ethylbenzthiazoline-6-sulfonate) (ABTS)/peroxidase (Kirkegaard
and Perry laboratories, Gaithersburg, MD) was used to develop the color, and 1% SDS was
added to stop the reaction. Optical density (OD) value was calculated by OD in ORF2 protein
coated well subtract OD in WT protein coated well. Antibody levels were reported as the
mean optical density (OD). The mean OD value from the infected group and non-infected
control group was compared.
Statistical analyses

In order to normalize the data for statistical comparisons, the Cap mRNA copy numbers and viral DNA copy numbers obtained from the real-time RT-PCR and real-time PCR, respectively, were log_{10} transformed. Data were assessed by analysis of variance (ANOVA) using JMP5.1 (SAS Institute Inc., Cary, NC). Differences were considered significant for all test procedures when P<0.05. Differences between groups were evaluated with Tukey's test. The correlation of the copy numbers of PCV2 Cap mRNA and copy numbers of viral DNA was evaluated by nonparametric correlations test, Spearman’s Rho in Multivariate Methods using JMP5.1.

Results

Quantification of PCV2 Cap mRNA and viral DNA from different tissues

To investigate PCV2 replication and distribution in infected pigs during the early stages of infection, PCV2 DNA and the Cap mRNA levels were measured at 3, 7, 14, and 21 DPI, and the results are shown in Fig. 1. At 3 DPI, no Cap mRNA was detected from any tissues with the exception of lung tissue from one infected pig whereas PCV2 DNA was detected in most tissues. However, there was no significant difference in levels of the viral DNA between the tested tissue samples at 3 DPI. The Cap mRNA was detected in most tissues at 7 DPI with the level in BLN being significantly higher than kidney and liver. No Cap mRNA was detected in thymus at that time. Viral DNA was present in all tissues and the level in BLN was significantly higher than kidney and thymus at 7 DPI. At 14 DPI, both the Cap mRNA and viral DNA were elevated in infected pigs. At that time, Cap mRNA was detected in all collected tissues and the level in the BLN was significantly higher than kidney and thymus. Levels of Cap mRNA in the ILN, spleen, lung, liver, and tonsil were equivalent
while the levels of Cap mRNA in the thymus were significantly lower than all other tissues. The level of PCV2 DNA at 14 DPI was consistent with the Cap mRNA levels with BLN level being significantly greater than the level in kidney, liver and thymus. At 21 DPI, the Cap mRNA levels were reduced in all tissues and no PCV2 Cap mRNA was detected in ILN, kidney, liver and thymus. In contrast, PCV2 DNA levels, while reduced compared to samples collected at 14 DPI, remained detectable in all tissues at 21 DPI. No Cap mRNA or viral DNA was amplified in any tissue samples from the negative control pigs at any time point during this experiment.

Strong correlation was found between the level of PCV2 Cap mRNA and the level of viral DNA in the following tested tissues: BLN ($r=0.75$, $P=0.005$), ILN ($r=0.82$, $P=0.001$), kidney ($r=0.82$, $P=0.001$), liver ($r=0.83$, $P=0.001$), lung ($r=0.92$, $P<0.0001$), spleen ($r=0.92$, $P<0.0001$), tonsil ($r=0.93$, $P<0.0001$)) except the thymus.

**Investigation of PCV2 Cap mRNA and viral DNA levels in PBMCs and BAL cells and the PCV2 DNA level in serum and cell-free BAL fluid**

To evaluate PCV2 infection and replication in the respiratory tract, the level of Cap mRNA and viral DNA was evaluated in BAL cells. Because PCV2 Cap mRNA as the transcript are cell associated, only viral DNA was assessed in the cell-free BAL fluid and serum. As shown in Fig. 2, Cap mRNA was detected in BAL cells 3 DPI from 2 of 3 infected pigs. The mean log$_{10}$ copy number of Cap mRNA increased from 2.87 per $1 \times 10^6$ cells at 3 DPI to 7.84 per $1 \times 10^6$ cells at 7 DPI and 7.24 per $1 \times 10^6$ cells at 14 DPI, followed by a decrease to 2.58 per $1 \times 10^6$ cells at 21 DPI. However, none of these differences in the mRNA levels were statistically significant between different time points. The level of PCV2 DNA in BAL cells began to increase at 7 DPI, was further increased at 14 DPI. That was followed by
a decreased level at 21 DPI. In the case of viral DNA, the levels in the cell fraction of the BAL were significantly greater at 7 and 14 DPI compared to 3 and 21 DPI. The levels of PCV2 DNA in cell-free BAL fluid were similar to the levels observed in BAL cells. A strong correlation was found between the level of Cap mRNA and the viral DNA in BAL cells ($P<0.001$) and between the level of PCV2 DNA in BAL cells and in cell-free BAL fluid ($P<0.001$).

To assess the presence of PCV2 infection and replication systemically, serum and PBMCs were collected at 2, 6, 13, and 20 DPI. As shown in Fig. 3, PCV2 Cap mRNA was detected in PBMCs only at 13 DPI, while the viral DNA was detected as early as 6 DPI. The viral DNA level was significantly higher in PBMCs at 13 DPI compared to 6 and 20 DPI. The detection of viral load in serum revealed a similar trend as that observed in PBMCs. A strong correlation was found between the level of Cap mRNA and the viral DNA in PBMCs ($P=0.004$). Moreover, a strong correlation was also found between the level of PCV2 DNA in PBMCs and in serum ($P<0.001$). No Cap mRNA or viral DNA was amplified in BAL cells or PBMCs and no PCV2 DNA was amplified in BAL fluid or serum samples from control pigs at any time point during the experiment.

Investigation of PCV2 infection and replication in mononuclear cell subpopulations isolated from peripheral blood or BLN from infected pigs

To investigate PCV2 replication in specific cell populations in vivo, T and B lymphocyte and monocyte populations were sorted from PBMCs collected at 13 DPI or BLN mononuclear cells collected at 7, 14, and 21 DPI from infected pigs. Cap mRNA and viral DNA were measured in each cell fraction. Purity of the T lymphocyte, B lymphocyte, and monocyte populations was confirmed by flow cytometry and was determined to be equal to
or greater than 95%. The viability of each purified population was determined to be greater than 95% (data not shown). As shown in Fig. 4, the level of Cap mRNA in T lymphocytes in PBMCs at 13 DPI was significantly higher compared to levels in monocytes ($P<0.05$). In contrast, the PCV2 DNA levels in B lymphocytes was significantly higher than the level in the monocyte fraction ($P<0.05$).

As shown in Fig. 5A, similar to the findings in PBMCs, the level of Cap mRNA in T or B lymphocytes were significantly higher than the level in monocytes ($P<0.001$) at 7 DPI. At 14 DPI, the level of Cap mRNA in B lymphocytes was significantly higher than the level in either T lymphocytes or monocytes ($P<0.05$). By 21 DPI, no statistical significant difference between the Cap mRNA levels in tested populations was present. The level of PCV2 DNA in each cell population is shown in Fig. 5B. There was no significant difference between the viral DNA levels in tested cell populations at 7 DPI. At 14 DPI, the amount of PCV2 DNA in B lymphocytes and monocytes was significantly increased compared to the level in T lymphocytes ($P<0.05$), whereas the amount of viral DNA was significantly higher in monocytes than either lymphocyte population at 21 DPI ($P=0.001$).

**Evaluation of Histopathology and Immunohistochemistry**

No clinical signs of disease were detected in any of the pigs throughout the study period. There were no microscopic lesions consistent with PCV2 infection in the tissues collected from the negative pigs at any point throughout the study. The mean microscopic lesion scores in the collected tissue samples from PCV2 infected pigs are summarized in Table 1. Microscopic lesions characterized by lymphoid depletion of follicles with macrophage infiltration were observed in BLN, ILN, and tonsils from 2 of 3 of the infected pigs as early as 7 DPI. At 14 DPI, characteristic microscopic lesions were observed in the
liver, BLN, tonsil, lung and ILN from all 3 tested pigs as well as in spleen and kidney from 2 of the 3 necropsied pigs. There was a significant difference \( (P<0.05) \) in the severity of PCV2-associated lesions observed in liver at 14 DPI. The severity of PCV2-associated lesions observed in BLN was significantly higher at 14 DPI than at 21 DPI. No microscopic lesions were observed in the lungs at 3 and 7 DPI whereas the lung lesion score was high at 14 DPI. The lesion scores of spleen, kidney, tonsil and ILN from infected pigs showed no significant differences at any time point throughout the trial. No microscopic lesions were observed in thymus samples from infected pigs at any time.

PCV2 antigen was detected by IHC in BLN and tonsils from 3 pigs, in spleen and lung from 2 of 3 pigs, and in thymus and ILN from 1 of 3 pigs at 7 DPI. At 14 DPI, PCV2 antigen was detected in liver, BLN, tonsils and lungs from all 3 pigs examined as well as in thymus, spleen and ILN from 2 of 3 pigs. The PCV2 antigen was still detectable in BLN from all 3 pigs and in the tonsils from 2 of the 3 pigs at 21 DPI. There was no detection of PCV2 antigen in kidney samples collected from infected pigs at any time point. There were no significant differences between the amounts of PCV2 antigen in thymus, spleen, or ILN collected from infected pigs at any necropsy day. At 14 DPI, the amount of PCV2 antigen in BLN was significantly \( (P<0.05) \) higher than that at 7 or 21 DPI. No immunostaining was detected in sections of collected tissues from the negative control pigs inoculated with PK-15 cell lysate at any time points.

The correlation was examined between the level of Cap mRNA or viral DNA and the amount of PCV2 antigen within each tested tissue except kidney in which no PCV2 antigen was detected. Strong correlations were revealed between the level of Cap mRNA and PCV2 antigen in liver \( (r=0.82, P=0.001) \), BLN \( (r=0.73, P=0.007) \), spleen \( (r=0.82, P=0.001) \), lung
(r=0.84, P=0.001), tonsil (r=0.87, P=0.0002), and ILN (r=0.71, P=0.009) but not in thymus (r=0.52, P=0.08). Strong correlations were also revealed between the level of PCV2 DNA and PCV2 antigen in liver (r=0.75, P=0.0046), thymus (r=0.59, P=0.04), BLN (r=0.90, P<0.0001), spleen (r=0.67, P=0.017), lung (r=0.88, P=0.0002), and tonsil (r=0.92, P<0.0001) but not in ILN (r=0.53, P=0.07). Except in the kidney tissues where no PCV2 antigen was detected, strong correlations were revealed between the level of PCV2 DNA in serum or in PBMCs and the amount of PCV2 antigens in all tested tissues, whereas no correlation was found between the level of Cap mRNA in PBMCs and the amount of PCV2 antigens in thymus, spleen or ILN (data not shown).

**Antibody response against PCV2**

Upon arrival, all pigs had maternal antibodies to PCV2 which decayed gradually. PCV2 inoculum was administered to 12 pigs at 4 weeks of age when antibody levels in those pigs were negative for PCV2 by ELISA using a positive cut-off level (S/P ratio 0.2) (Nawagitgul et al., 2002). The remaining 12 pigs with antibody levels above the cut-off level were placed in the negative control group and sham-infected. At 3 DPI, serum samples from the negative control pigs were still PCV2 antibody positive, but all pigs were seronegative by 7 DPI and remained negative for PCV2 antibody until the completion of the study. No seroconversion to PCV2 was observed in infected pigs until 21 DPI, the last day of this experiment (data not shown). An ELISA assay was performed to detect PCV2-specific antibodies in BAL. No significant differences in the PCV2-specific IgG level in BAL fluid were detected between the PCV2 infected group and the negative control group at any time throughout the trial (data not shown). As shown in Fig. 6, the PCV2-specific IgM antibody levels were increased from...
7 DPI in BAL fluid from infected pigs and the levels were significantly higher compared to the negative control pigs at 14 and 21 DPI. The level of PCV2-specific IgM antibodies was significantly higher in BAL from control pigs than from infected pigs at 3 DPI. This may be due to the effect of remaining maternal antibody in those pigs. Similar findings were observed with PCV2-specific IgA antibodies at 3 DPI, with the negative control group having higher levels than the infected group. The PCV2-specific IgA antibody levels in BAL fluid were increased in the infected group at 14 DPI, although no statistical difference was present. At 21 DPI, the level of PCV2-specific IgA antibody was significantly higher in the BAL fluid from infected pigs compared to the control pigs (Fig. 7).

A strong negative correlation was found between the PCV2-specific IgA antibody levels and Cap mRNA or viral DNA levels in BAL cells, or the level of PCV2 DNA in cell-free BAL fluid from infected pigs at 14 DPI. A strong negative correlation was also found between the PCV2-specific IgM antibody level and the level of PCV2 DNA in BAL cells or the cell-free BAL fluid from infected pigs at 21 DPI.

Discussion

The goal of this study was to investigate PCV2 infection and replication early in infection and identify cell populations that are permissive for PCV2 replication in vivo. This study was performed on conventional pigs that had been inoculated with PCV2 by the intranasal route, which is potentially a common route of exposure of young swine (Calsamiglia et al., 2002; Shibata et al., 2003; Yang et al., 2003). In an earlier study, a sensitive real-time RT-PCR assay was developed that enabled quantification of PCV2 Cap mRNA in infected PBMCs in vitro (Yu, et al., 2004). The results of this earlier study demonstrated that it was feasible to discriminate between replicating PCV2 and PCV2
virus. In the present study, viral replication was measured in multiple tissues and cells recovered from experimentally infected pigs at 3, 7, 14, and 21 DPI.

The results of this study determined that the level of viral DNA and the amount of Cap mRNA differed dramatically between tissues obtained from the same pig. Analysis of BLN samples consistently provided the highest yield of the Cap transcript and consistently higher levels of viral DNA than any other tissues tested at 7, 14, or 21 DPI. Because the pigs were inoculated using the intranasal route, BLN would be the lymphoid tissue closest to the inoculation site assessed in this study and as a result, had higher levels of both Cap mRNA and viral DNA levels compared to the more distant ILN. In addition, the amount of Cap mRNA in BLN samples peaked at 7 DPI while levels of Cap mRNA or viral DNA in ILN were greatest at 14 DPI. At 21 DPI, Cap mRNA was no longer detectable in the ILN while the amount of the Cap mRNA remained at a moderate high level in BLN. These findings suggested that the PCV2 begins replication in the lymph nodes nearest the site of infection and then spreads systemically. Moreover, the results of this study suggested that the lymph nodes near the infection site may play an important role in PCV2 persistence in the infected host.

Though viral DNA was detected in the thymus and there was positive staining by IHC for PCV2 antigen at 7 and 14 DPI, the amplification of the Cap mRNA was only detected from one infected pig at a statistically significantly reduced level at 14 DPI (P<0.05). These results indicate that the thymus may not be an important site for PCV2 replication. This is in contrast to an earlier study using 1-day old colostrum-deprived piglets that had been experimentally infected by oral/nasal and intravenous (IV) routes found that thymus was the primary site of PCV2 antigen detection (Allan et al., 1995). The differences observed
between that study and the data presented here may have been due to the IV challenge route in their study or due to differences in ages of the pigs, with the pigs in the present study being 4-5 weeks of age and from a conventional farm source. Another PCV2 and porcine parvovirus co-infection study also demonstrated that the thymus, like other lymph nodes and tonsil was the primary detection site of PCV2 DNA in co-infected 4-week old pigs by ISH (Kim et al., 2003). Whether the virus can replicate in thymic cells under certain conditions and why PCV2 antigen and DNA were detected there needs to be further investigated.

Assessment of PCV2 replication and viral DNA levels systemically was determined by evaluating serum and PBMCs at 2, 6, 13 and 20 DPI. It was demonstrated that Cap mRNA was detected in PBMCs only at 13 DPI, while the viral DNA was detected earlier at 6 DPI. In addition, the level of viral DNA was significantly higher at 13 DPI compared with the levels at 6 or 20 DPI. As the Cap mRNA is the replication product of PCV2, this data indicates that PCV2 is able to replicate in PBMCs in vivo, though it may be a transient process. These findings suggest that PBMCs may not be a major site for PCV2 replication but may play an important role in viral dissemination. In this study, PCV2 DNA levels in serum and the amount of viral DNA and Cap mRNA in PBMCs correlated well with the amount of viral DNA in all tested tissues. These findings are important as high levels of PCV2 appear to be crucial in the pathogenesis of PMWS (Allan et al., 2000b; Krakowka et al., 2005). In the present study, it was shown that the Cap mRNA in PBMCs was only detected when the microscopic lesions were most severe and the highest levels PCV2 antigens were present in tissues. Therefore, these findings suggest that the detection of PCV2 Cap mRNA in PBMCs from infected pigs may be predictive of disease progression in vivo.
PCV2 capsid antigens have been described in the cytoplasm of monocytes and alveolar macrophages (Allan et al., 1994). It has been questioned whether this signal was due to transcription of the viral genome or due to accumulation of virus present in other cell populations that was taken up by phagocytosis. Previous in vitro studies demonstrated that PAMs may not be a primary cell type for PCV2 replication (Gilpin et al., 2003; Meerts et al., 2005). In contrast to their findings, Cap mRNA was detected in BAL cells as early as 3 DPI from a pig which had a high level of the transcription product in lung tissue in the present study. Over time, the levels increased and remained high throughout the period of the study. A similar trend was observed with the levels of PCV2 DNA in BAL cells. Because cells from BAL consisted of greater than 90% pulmonary alveolar macrophages (PAMs) (Ganter and Hensel, 1997), PAMs appears to be an important cell type for PCV2 replication in infected pigs under certain conditions. However, the ability to detect high levels of PCV2 DNA and its replication product in BAL cells in our study needs further investigation, especially under different stimulation factors of PCV2 infection in vivo.

PCV2 replication was assessed in specific lymphocyte and monocyte populations separated from PBMCs collected at 13 DPI or from BLN mononuclear cells at 7, 14, and 21 DPI from infected pigs. In contrast to previous findings that lymphocytes appear not to be the primary site for PCV2 replication (Allan and Ellis, 2000; Gilpin et al., 2003; Rosell et al., 1999), the results of this study demonstrate that T and B lymphocytes are important cell populations that support PCV2 replication. These in vivo results support an earlier in vitro study in which it was demonstrated that PCV2 replicated in lymphocytes under ConA stimulation (Yu et al., 2004). In the present study, it was demonstrated that as the Cap mRNA and viral DNA levels in the lymphocytes from BLN mononuclear cells decreased at 21 DPI,
the level of viral DNA in monocytes remained consistent and as a result these cells become
the cell population with the highest amount of PCV2 DNA. These findings suggest that while
the lymphocytes appear to be primary sites for PCV2 replication, the monocytes may be the
site for PCV2 persistence in the infected host.

Previous studies have suggested there is an immunosuppressive feature associated
with PMWS due to the lack of response to antibiotic therapy in systemic and/or pulmonary
bacterial infections in PMWS affected farms (Segales et al., 2004a; Segales et al., 1997) as
well as infections with unusual swine pathogens, such as *Pneumocystis carinii* (Darwich et
al., 2004), *Chlamydia* spp. *Aspergillus* spp and *Cryptosporidium parvum* (Carrasco et al.,
2000; Clark, 1997; Ellis et al., 1998; Nunez et al., 2003; Segales et al., 2004a). Furthermore,
pathologic, immunohistologic, and flow cytometric studies have shown that lymphocyte
depletion of follicular and interfollicular areas, together with macrophage infiltration of
lymphoid tissues presented as a unique lesion observed in PMWS affected pigs (Chianini et
al., 2003; Clark, 1997; Darwich et al., 2002; Quintana et al., 2001; Rosell et al., 1999; Sarli et
al., 2001; Shibahara et al., 2000) and is highly correlated with a decrease in circulating B and
T cells (Darwich et al., 2002; Nielsen et al., 2003; Segales et al., 2000). Like chicken anemia
virus, classical swine fever virus, porcine reproductive and respiratory syndrome virus
(PRRSV) and the human immunodeficiency virus (HIV), all of which infect immune cells
and cause immunosuppression in the infected host (Markowski-Grimsrud and Schat, 2003;
Pauly et al., 1998; Shellito, 2004), the present observations demonstrating that PCV2
infection and active replication in immune cell populations suggest a similar pathogenic
mechanism by which PCV2 may induce immunosuppression that has been associated with
PMWS.
The absence of clinical signs in the PCV2 inoculated pigs in this study indicates that the PCV2 infection alone was subclinical and suggests that an additional factor is needed for the reproduction of PMWS disease. Similar findings have been observed in a number of earlier studies indicating that the co-factors such as porcine parvovirus (PPV), PRRSV, *Mycoplasma hyopneumoniae*, or immunostimulating factors are needed to develop the full spectrum of clinical PWMS (Allan et al., 2000a; Harms et al., 2001; Kennedy et al., 2000; Krakowka et al., 2001; Krakowka et al., 2000; Kyriakis et al., 2002; Opriessnig et al., 2003; Rovira et al., 2002 ). Several studies have demonstrated that there is a correlation between the amount of PCV2 antigen or genetic material detected in the tissue and the severity of the lymphoid depletion associated with PMWS (Chianini et al., 2003; Darwich et al., 2002; Rosell et al., 1999). However, this correlation was only demonstrated for samples from BLN and liver and was not demonstrable for samples from other tissues analyzed in this study. These results may be due to the lack of an additional factor. Thus, the amount of virus detected in PCV2 infected pigs was less than what has been reported in pigs with clinical PMWS. In contrast to the previous study in which the ILN had more severe lesions compared to other lymphoid tissues in field affected-PMWS pigs (Calsamiglia et al., 2002; Sarli et al., 2001), an increased severity of lesions was demonstrated in BLN which was the lymph node closest to the inoculation site. Whether PCV2 replication and persistence is related to the exposure area or the active status of local lymph node needs to be further studied.

With exception of the kidneys in which no PCV2 antigens were detected and the thymus, which lacked the replication product in this study, there were strong correlations between the level of Cap mRNA and the level of viral DNA or the amount of PCV2 antigen.
In addition, a strong correlation was observed between the level of viral DNA and PCV2 antigen in liver, thymus, BLN, spleen, lung, and tonsil but not in ILN.

In this study, no seroconversion occurred in PCV2 infected pigs at 2, 6, 13, or 20 DPI. No significant difference was detected in the IgG level in BAL fluid between the PCV2 infected group and the negative control group over time. However, PCV2-specific IgA and IgM were detected in BAL fluid at 14 and 21 DPI. In addition, a strong negative correlation was found between the level of IgA antibody and the amount of Cap mRNA or viral DNA in BAL cells, or the level of PCV2 DNA in cell-free BAL fluid at 14 DPI. A strong negative correlation was also found between the IgM antibody levels and the level of PCV2 DNA in BAL cells or the BAL cell-free fluid from infected pigs at 21 DPI. These findings suggest that the IgG response to PCV2 occurs late in the infection process and the local antibody responses (e.g., IgA and IgM), but not systemic IgG may be important in controlling PCV2 during early infection. In our study, higher levels of IgA and IgM in BAL were observed in the negative control pigs at 3 DPI and may be attributed to maternal antibodies. As antibody levels in the negative control pigs continued to decrease over time, the PCV2-specific IgA and IgM antibody levels in the infected pigs began to increase after 7 DPI. The presence of PCV2-specific IgA and IgM in the BAL associated with maternal antibodies may play a role in their reported protection in young pigs {Allan, 2002 #334}.

The results of this study demonstrated that PCV2 replication as measured by Cap mRNA occurs in multiple tissues but with highest levels in BLN of pigs in the early stages of infection. There was a strong correlation between the level of this transcript and viral DNA levels in all tested tissues except the thymus in which no Cap mRNA was detected at 3, 7 and 21 DPI. In addition, we demonstrated that it was possible to detect PCV2 Cap mRNA in
PBMCs from infected pigs which may indicate active viral replication in these cells *in vivo*.

More importantly, the results of this study confirmed the earlier findings that PCV2 replicates in T lymphocytes and further demonstrated that B cells appear to be the primary site for PCV2 replication during early infection (Yu et al., 2004). The present findings will increase our knowledge of the cell populations and tissues in which PCV2 replicated following challenge and further the understanding of the pathogenesis of PCV2 infection.

**Acknowledgements**

The authors would like to thanks Dr. Xiang-Jin Meng from Virginia Polytechnic Institute and State University for supplying the PCV2 plasmid DNA, and Wasin Charerntantanakul for giving some advice on the statistical analysis of the data. This work was supported by the Healthy Livestock Initiative fund.

**References**


Table 1. Microscopic lesions of tissue samples in PCV2 infected pigs at 3, 7, 14 and 21 days post-infection (DPI).

<table>
<thead>
<tr>
<th>DPI</th>
<th>liver</th>
<th>thymus</th>
<th>BLN</th>
<th>spleen</th>
<th>kidney</th>
<th>tonsil</th>
<th>lung</th>
<th>ILN</th>
</tr>
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<tbody>
<tr>
<td>3</td>
<td>0.33±0.58&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.00±0.00</td>
<td>0.67±0.58&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
<td>0.33±0.58</td>
<td>0.00±0.00</td>
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</tr>
<tr>
<td>7</td>
<td>0.33±0.58&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.00±0.00</td>
<td>1.33±1.15&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
<td>0.67±0.58</td>
<td>0.00±0.00&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.66±0.58</td>
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<tr>
<td>14</td>
<td>1.67±0.58&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.00±0.00</td>
<td>3.00±0.00&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.67±0.58</td>
<td>0.67±0.58</td>
<td>1.00±0.00&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.33±0.58&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>21</td>
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<td>0.00±0.00</td>
<td>1.00±0.00&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.33±0.58</td>
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<td>0.33±0.58&lt;sup&gt;b&lt;/sup&gt;</td>
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</table>

Tissue samples are bronchial lymph nodes (BLN), superficial inguinal lymph nodes (ILN), kidney, liver, lung, spleen, thymus, and tonsil.

Different superscripts (a, b) within each column indicate that values in mean score within infection group for each DPI was significantly different \( (P<0.05) \).

Values are presented as mean score ± standard deviation. Scoring: 0 = normal; 1 = mild; 2 = moderate; 3 = severe.
Table 2. Immunohistochemical analysis of tissue samples from infected pigs for the presence of PCV2 antigen.

<table>
<thead>
<tr>
<th>DPI</th>
<th>liver</th>
<th>thymus</th>
<th>BLN</th>
<th>spleen</th>
<th>kidney</th>
<th>tonsil</th>
<th>lung</th>
<th>ILN</th>
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<tr>
<td>3</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
<td>0.00±0.00c</td>
<td>0.00±0.00</td>
<td>0.00±0.00b</td>
<td>0.00±0.00b</td>
<td>0.00±0.00b</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td>7</td>
<td>0.00±0.00a</td>
<td>0.33±0.57</td>
<td>1.67±0.58b</td>
<td>0.67±0.58a</td>
<td>0.00±0.00a</td>
<td>1.00±0.00ab</td>
<td>0.67±0.58ab</td>
<td>0.33±0.58</td>
</tr>
<tr>
<td>14</td>
<td>1.00±0.00a</td>
<td>0.66±0.57</td>
<td>3.00±0.00a</td>
<td>0.67±0.58a</td>
<td>0.00±0.00a</td>
<td>1.67±0.58a</td>
<td>1.33±0.58a</td>
<td>0.67±0.58</td>
</tr>
<tr>
<td>21</td>
<td>0.00±0.00a</td>
<td>0.00±0.00</td>
<td>1.67±0.58b</td>
<td>0.00±0.00b</td>
<td>0.00±0.00b</td>
<td>0.67±0.58ab</td>
<td>0.00±0.00b</td>
<td>0.00±0.00</td>
</tr>
</tbody>
</table>

Tissue samples are bronchial lymph nodes (BLN), superficial inguinal lymph nodes (ILN), kidney, liver, lung, spleen, thymus, and tonsil from PCV2 infected 4-week-old conventional pigs at 3, 7, 14 and 21 days post-infection (DPI).

Different superscripts (a, b, c) within each column indicate that values in mean score within infection group for each DPI were significantly different ($P<0.05$).

Values are presented as mean score ± standard deviation. Scoring: ranging from 0 = not detectable to 3 = strong signal.
Fig. 1. Quantification of PCV2 spliced capsid mRNA (A) and PCV2 DNA (B) in tissue samples from infected pigs at 3, 7, 14 and 21 days post-infection (DPI). Tissue samples are bronchial lymph nodes (BLN), superficial inguinal lymph nodes (ILN), kidney, liver, lung, spleen, thymus, and tonsil. Averages with different letters (a, b, c) within the tissues at each DPI are statistically different by least significant difference (P<0.05). Values in Fig. 1A are expressed as logarithm of PCV2 spliced capsid mRNA copies/mg of tissue. Values in Fig. 1B are expressed as logarithm of PCV2 DNA copies/mg of tissue.
Fig. 2. Quantification of PCV2 DNA in cell-free bronchoalveolar lavage (BAL) fluid or BAL cells, and PCV2 spliced capsid mRNA in BAL cells. Averages with different letters (a, b) within the levels of nucleic acid products in tested samples at each DPI are statistically different by least significant difference ($P<0.05$). Values are expressed as logarithm of PCV2 DNA copies/ml of BAL fluid, or $10^6$ cells and as logarithm of PCV2 spliced capsid mRNA copies/$10^6$ cells.
Fig. 3. Quantification of PCV2 DNA in serum or peripheral blood mononuclear cells (PBMCs), and PCV2 spliced capsid mRNA in PBMCs. Averages with different letters (a, b) within the levels of nucleic acid products in tested samples tissues at each DPI are statistically different by least significant difference ($P<0.05$). Values are expressed as logarithm of PCV2 DNA copies/ml of serum, mg of tissue or $10^6$ cells and as logarithm of PCV2 spliced capsid mRNA copies/$10^6$ cells.
Fig. 4. Quantification of PCV2 spliced capsid mRNA and viral DNA in purified cell populations from peripheral blood mononuclear cells (PBMCs) from infected pigs at 13 days post-infection (DPI). Purified cell populations are T lymphocytes (T), B lymphocytes (B), and monocytes (Mono+). Averages with different letters (a, b) within the cell fractions are statistically different ($P<0.05$). Values are expressed as logarithm of PCV2 DNA copies/10$^6$ cells and logarithm of PCV2 spliced capsid mRNA copies/10$^6$ cells.
Fig. 5. Quantification of PCV2 spliced capsid mRNA (A) and PCV2 DNA (B) in purified cell populations from bronchial lymph node mononuclear cells from infected pigs. Purified cell populations are T lymphocytes (T), B lymphocytes (B) and monocytes (Mono+). Averages with different letters (a, b) within the cell fractions at each day post-infection (DPI) are statistically different by least significant difference ($P<0.05$). Values are expressed as logarithm of PCV2 DNA copies/10⁶ cells and logarithm of PCV2 spliced capsid mRNA copies/10⁶ cells.
Fig. 6. Comparison of PCV2 specific IgM antibody levels in bronchoalveolar lavage fluid from PCV2 infected and non-infected groups. Samples are collected at 3, 7, 14 and 21 days post-infection (DPI). The symbol * represents significant difference between PCV2 infected group and negative control group within each DPI at $P<0.05$.

Fig. 7. Comparison of PCV2 specific IgA antibody levels in bronchoalveolar lavage fluid from PCV2 infected and non-infected groups. Samples are collected at 3, 7, 14 and 21 day post-infection (DPI). The symbol * represents significant difference PCV2 infected group and negative control group within each DPI at $P<0.05$. 
CHAPTER 5. THE EFFECTS OF PCV2 INFECTION AND REPLICATION ON ACTIVATED PORCINE PERIPHERAL BLOOD MONONUCLEAR CELLS IN VITRO AND THE RELATIONSHIP BETWEEN PCV2 REPLICATION AND CELLULAR PROLIFERATION

A paper to be submitted to Veterinary Research

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Abstract

This study was to examine the effect of porcine circovirus type 2 (PCV2) infection and replication on peripheral blood mononuclear cells (PBMCs) in the presence of the mitogens, concanavalin A (ConA) or pokeweed mitogen (PWM) in vitro as well as the relationship between PCV2 replication and cell proliferation. PCV2 replication, cell viability and stimulation, and apoptosis indices in PCV2 infected PBMCs with or without mitogen stimulation were measured. The data showed that mitogen stimulation increased viral replication. PCV2 infection did not affect the ability of PBMCs to proliferate in response to ConA or PWM stimulation as no significant differences in proliferation occurred in PCV2 infected PBMCs with different stimuli in vitro. Increased apoptosis was associated with PCV2 infection in PWM stimulated PBMCs. Interestingly, a significantly lower apoptotic index (AI) was observed in PCV2 infected PBMCs compared to non-infected cells in the
absence of mitogens. No significant differences were observed in the levels of PCV2 replication product, the spliced capsid mRNA (Cap mRNA), in PBMCs stimulated with ConA for 12, 36, and 72 hours prior to infection. It was also demonstrated that there was no significant differences in the amount of Cap mRNA in ConA or PWM stimulated proliferating PBMCs compared to non-proliferating cells. In conclusion, the findings of this study indicated that PCV2 replication increases with cell stimulation and apoptosis increased with PCV2 infection under certain stimulation conditions. In addition, our results suggest that PCV2 needs a specific stimulation/trigger to enhance viral replication, which is not dependent on cell division and proliferation.

**Keywords:** PCV2; Peripheral blood mononuclear cells; Proliferation; Apoptosis

**Introduction**

Porcine circovirus type 2 (PCV2) is a virus in the *Circoviridae* family and is the cause of postweaning multisystemic wasting syndrome (PMWS) [30, 6]. Lesions associated with PCV2 infection are characterized by lymphocyte depletion of follicular and interfollicular areas together with macrophage infiltration of lymphoid tissues [1]. These findings are highly correlated with a decrease in circulating B and T cells and reduced numbers of lymphocytes in lymphoid organs [8, 10, 22, 27, 32, 33].

A decrease in the number of B lymphocytes and high number of apoptotic B lymphocytes along with macrophages containing PCV2 antigen and virions have been reported in lymphoid tissues of pigs with clinical disease consistent with PMWS [33]. This study suggested that PCV2 infection induces apoptosis in B lymphocytes, followed by phagocytosis by macrophages resulting in lymphoid depletion. However, other studies appear to contradict these results by finding reduced levels of apoptosis in the B-cell areas of
lymphoid tissues of PMWS affected pigs compared to healthy non-PCV2 infected pigs [25]. Apoptosis associated with systemic viral diseases can be attributed to pyrexia in addition to the direct or indirect effects of viruses on target cells [16]. Decreased cell proliferation and not increased apoptosis appeared to be an important factor in the depletion of cells in lymphoid tissues from PMWS-affected pigs under field conditions [20]. Research has suggested that vaccination and stimulation of an immune response by antigens or adjuvants may predispose pigs to PMWS [15, 17, 18]. Vaccination or any stimulation of the immune system could stimulate lymphocytes to proliferate and enhance the replication of PCV2 in lymphocytes as they divide. These conflicting theories make the pathogenesis of the lymphoid depletion associated with PCV2 infection confusing. Several studies have demonstrated a correlation between the amount of PCV2 antigen or genetic material detected in tissues and the severity of the lymphoid depletion [8, 10, 26]. A recent study in our laboratory demonstrated that PCV2 replication in infected PBMCs was significantly increased when PBMCs were stimulated with ConA compared to unstimulated PBMCs [39]. The aim of this study was to evaluate the effect of PCV2 infection on activated porcine PBMCs in vitro by measuring proliferation, cell viability and apoptosis as well as the level of PCV2 replication and cellular proliferation. Previous study showed that PCV2 spliced capsid mRNA (Cap mRNA) increases in a time dependent manner during PCV2 replication in PK-15 cells [7]. Previously, it was demonstrated that PCV2 Cap mRNA can be used as a measure of PCV2 replication [38, 39]. A real-time reverse transcription polymerase chain reaction (RT-PCR) assay to detect PCV2 Cap mRNA was used to assess viral replication in PBMCs in vitro in this study. Understanding the impact of PCV2 infection on stimulated immune cells will increase our knowledge of the pathogenesis of PCV2 infection.
Materials and methods

Preparation of PCV2

PCV2 isolate 40895 (GenBank Accession No. AF264042) previously shown to induce PMWS in pigs [13] and passaged in PK-15 cells was used in this study. The titer of the virus was $1 \times 10^4$ 50% tissue culture infectious dose (TCID$_{50}$/ml as determined by titration on PK-15 cells using an immunofluorescence assay (IFA) [13]. All PK-15 cells used in this study were confirmed to be PCV-free [13].

Experimental design

To prepare PBMCs, blood samples were collected by jugular venipuncture in heparinized tubes from four healthy PCV2-negative crossbred pigs. The PBMCs were isolated by density gradient centrifugation [37] followed by labeling with 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (CFSE) (Molecular Probes, Carlsbad, CA), a cytoplasmic dye that is used to measure cell division as described previously [19] with modifications. Briefly, $1 \times 10^6$ PBMCs suspension from each pig was centrifuged in a 15-ml conical tube. The supernatant was decanted, and the pellet resuspended in 1 ml of sterile phosphate-buffered saline (PBS). One ml of CFSE at a concentration of 2.0 μm was added to each tube and vortexed. Cells were incubated at room temperature for 5 min with periodic mixing and sterile PBS was added to bring the final volume to 10 ml following the addition of 0.5 ml of fetal bovine serum (FBS) to each tube to absorb excess CFSE. The cells were centrifuged for 10 min at 4 °C to remove the FBS from the media. Cells were resuspended in 10 ml of complete media consisting of RPMI 1640 supplemented with 10% fetal calf serum, 0.05 mM 2-mercaptoethanol, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 1% non-essential amino acids 100x (GibcoBRL, Life Technologies, Carlsbad, CA), and 2 mM L-glutamine.
(Sigma, St. Louis, MO) followed by centrifuging and resuspension in 500 µl complete media. Following CFSE labeling, cells were counted, infected with PCV2 at a multiplicity of infection (MOI) of 0.1 for one hour and incubated in 96-well plate with or without ConA (5 µg/ml) or PWM (10 µg/ml). Control cells were treated identically without the addition of PCV2 and/or mitogen. Cells were harvested at 3 and 5 days post-infection (DPI) and were enumerated after the addition of a non-enzymatic cell dissociation solution (Sigma-Aldrich, St. Louis, MO). Cell viability was determined using trypan blue exclusion. An aliquot containing 2×10^5 viable cells/sample was centrifuged and the cell pellet was suspended in 200 µl RNAlater (Ambion, Austin, TX) and used for total RNA isolation.

Lymphocyte proliferation of CFSE-labeled PBMCs infected with PCV2 was assessed with or without mitogen stimulation. CFSE-labeled PBMCs were harvested at 3 and 5 days following stimulation with ConA (5 µg/ml) or PWM (10 µg/ml). Flow cytometry was used to detect CFSE intensity as previously described [21, 14]. ModFit software (Verity Software House, Topsham, ME) was used to analyze the CSFE intensity of cells to determine the cell proliferation index. PBMCs from each pig incubated with medium alone were used as the negative control. The stimulation index was calculated by sample proliferation index/negative control proliferation index.

To determine cell viability and apoptosis index in PCV2 infected PBMCs stimulated with ConA or PWM, PBMCs were cultured with each mitogen as described above and harvested at 3 and 5 DPI. The cells were washed and simultaneously labeled with FITC-Annexin V (AN) (BD Biosciences Pharmingen, San Jose, CA) and 7-amino-actinomycin D (7AAD) (BD Biosciences Pharmingen, San Jose, CA) according to the company’s protocol. Flow cytometric analysis of labeled cells was performed in a FACScan (Becton-Dickinson,
Franklin Lakes, NJ) using CellQuest (BD Biosciences, San Jose, CA). The AN-/7AAD-, AN+/7AAD-, and AN+/7AAD+ populations have been found to correspond to live cells, early apoptotic cells and late apoptotic and necrotic cells, respectively [34]. The apoptotic index (AI) was calculated as follows: (% of Annexin V+ cells) / (% of Annexin V- cells) + (% of Annexin V+ cells) when cells are gated by 7AAD negative staining.

To determine whether cellular proliferation increases PCV2 replication, PBMCs were stimulated with ConA (5 μg/ml) for 12, 36, and 72 hours prior to inoculation with PCV2 at a MOI of 0.1. The cells were counted and viability assessed by trypan blue exclusion at 24 hours post-infection (HPI). An aliquot of 1×10^6 viable cells was centrifuged and the pellet suspended in 200 μl RNAlater for total RNA isolation.

To determine the effect cell proliferation has on PCV2 replication, PBMCs were isolated and labeled with CFSE as described above. The CFSE labeled, PCV2 infected cell samples were harvested at 5 days post ConA or PWM stimulation. Proliferated cells (low CFSE intensity) and the non-proliferated cells (high CFSE intensity) were collected separately within the live cell gate using an EPICS ALTRA flow cytometer (Beckman Coulter, Miami, FL) at the Iowa State University cell facility. Each cell fraction (2×10^5 cells/sample) was centrifuged and the pellet suspended in 200 μl RNAlater for total RNA isolation.

**Total RNA extraction**

Total RNA was extracted using the Versagene total RNA purification kit (Gentra Systems, Minneapolis, MN) according to the manufacturer’s instruction, and solubilized in 70 μl of elution solution. The RNA extracts were stored at −80 °C until real-time RT-PCR amplification was carried out.
Real-time RT-PCR assay

The forward primer (5'-AGATGCCATTTTTCTCTTCTC-3'), reverse primer (5'-GCTCCACATTCAATAACTATGAC-3') and the probe (5'-/56-FAM/TCTTCTTCTGCGGTACCGCCTC/3BHQ_1/-3') were used to quantify Cap mRNA of PCV2 in real-time RT-PCR assay. The probe was labeled with a fluorescent reporter dye, 6-carboxyfluorescein (FAM) at 5' end and a quencher dye, Black Hole Quencher 1 (BHQ_1) at 3' end. RNA amplification and quantification was performed on Rotor-Gene RG-300 (Corbett Research, Sydney, AU). The reaction mixture was in a 20 μl final volume and the reactions were carried out at 55 °C for 45 min, 95 °C for 10 min; then for 45 cycles at 95 °C for 15 sec and 60 °C for 60 sec. All samples and standard dilutions ranging from 500 to \(5 \times 10^6\) copy numbers were run in duplicates. Quantification of PCV2 Cap mRNA was achieved by comparing the threshold cycle (C_T) value of the input sample RNA with the C_T value of the standard RNA.

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey–Kramer multiple comparisons test. \(P<0.05\) was considered to be statistically significant for all test procedures. The correlation of the copy numbers of PCV2 Cap mRNA, stimulation index and apoptosis index were evaluated by nonparametric correlations test, Spearman’s Rho in Multivariate Methods using JMP5.1 (SAS Institute Inc., Cary, NC).
Results

Quantification of the Cap mRNA in PCV2 infected PBMCs stimulated with ConA or PWM

The levels of Cap mRNA were assessed in PBMCs collected at 3 and 5 DPI (Table 1). The level of Cap mRNA was significantly greater in PBMCs with either ConA or PWM stimulation compared to non-stimulated cells at both time points. No significant differences in the level of Cap mRNA in infected PBMCs were observed between ConA and PWM stimulated cells at 3 DPI. However, the level of Cap mRNA in PWM stimulated cells was significantly greater than in ConA stimulated cells at 5 DPI. No significant differences in the levels of Cap mRNA were observed between 3 DPI and 5 DPI within each treatment. No Cap mRNA was detected in non-infected PBMCs with or without ConA or PWM stimulation at either collection day.

Evaluation of cell proliferation

The ability of PCV2 infected PBMCs to proliferate in response to ConA or PWM stimulation at 3 and 5 DPI, CFSE intensity was measured and a cell proliferation index determined. No significant difference in the ability of cells to proliferate was observed between PCV2 infected and non-infected PBMCs at either time or with either mitogen (Table 2). The stimulation index in PCV2 infected PBMCs with ConA stimulation was greater at 5 days post-stimulation than 3 days, though no significant difference in the index was found in PCV2 infected PBMCs with PWM stimulation between those two days. No correlation was found between the copy numbers of PCV2 Cap mRNA and the stimulation index in any tested cell samples (data not shown).
Cell viability and apoptosis analysis

Cell viability and apoptosis were measured using Annexin V and 7AAD staining. As shown in Table 3, there were no significant differences in the percentage of live cells between PCV2 infected and non-infected PBMCs within each treatment group at either 3 or 5 DPI. However, cell viability was significantly lower in PCV2 infected PBMCs stimulated with PWM compared to cells without stimulation at 3 and 5 DPI or with ConA stimulation at 5 DPI. The results of the apoptotic index from tested samples demonstrated that PWM stimulated and PCV2 infected cells had a significantly higher apoptotic index compared to the non-infected PBMCs at 3 and 5 DPI. No significant difference was observed in ConA stimulated cells (Fig. 2A and Fig. 2B). Without mitogen stimulation, a significantly lower apoptotic index was detected in PCV2 infected PBMC samples at both 3 and 5 DPI. No significant correlation was found between the copy numbers of Cap mRNA and the apoptotic index in tested cell samples (data not shown).

Evaluation of the relationship between PCV2 replication and cellular proliferation

To study the effect of PBMC proliferation on PCV2 replication, the levels of Cap mRNA were assessed in PBMCs that had been stimulated with ConA for 12, 36, or 72 hours prior to inoculation of PCV2 for 24 hours. No significant differences were observed in the levels of Cap mRNA in PBMCs stimulated at any time point prior to PCV2 infection (data not shown).

To further determine the relationship between cellular proliferation and PCV2 replication, PCV2 infected PBMCs were labeled with CFSE and stimulated with ConA or PWM for 5 days. No significant differences in Cap mRNA copy numbers were observed in ConA or PWM stimulated PBMCs that had proliferated compared to non-proliferated cells.
However, the level of Cap mRNA was significantly greater in proliferated and non-proliferated cells stimulated with PWM compared to ConA stimulation (Fig. 1).

**Discussion**

PCV2 infection is associated with lymphoid depletion and histiocytic and granulomatous replacement of follicles in lymphoid tissues [2, 9]. It has been reported that the use of immunostimulating factors, such as keyhole limpet hemocyanin (KLH) emulsified in incomplete Freund's adjuvant, vaccines, or non-specific immunomodulating drugs (Baypamun, Bayer, Leverkusen, Germany) increases the amount of PCV2 antigen in tissues and can increase the number of pigs that develop PMWS [3, 17, 18, 23]. A consistent finding in pigs with PMWS is abundant PCV2 antigen in macrophages in the lymphoid tissues and the presence of a marker for late apoptosis characterized by terminal deoxynucleotidyl transferase (TdT)-mediated incorporation of biotinylated nucleotides (UTP) onto the 3'-exposed hydroxyl groups (nick end labeling) nuclear deoxyribonucleic acid (TUNEL) [33]. In contrast, recent studies suggested that PCV2 does not induce apoptosis of lymphocytes in PMWS affected pigs [16] and that decreased cell proliferation and not increased apoptosis appeared to be important mechanism for lymphoid depletion in PMWS-affected pigs under field conditions [20]. To date, little is known about the effect of PCV2 infection and replication on the immune cells of PWMS-affected pigs. The objective of this study was to evaluate the effects of PCV2 infection and replication on activated PBMCs in vitro. The virus replication, the stimulation index and apoptotic index in PCV2 infected PBMCs with or without stimuli were investigated.

Recently, we have developed an RT-PCR assay that detects PCV2 Cap mRNA and is able to discriminate between replicating PCV2 and PCV2 virions in PK-15 cells [38]. A
strong correlation between the levels of Cap mRNA and viral DNA in ConA stimulated PBMCs was observed [39]. In the present study, Cap mRNA was used to measure PCV2 replication in PCV2 infected PBMCs stimulated with ConA or PWM. ConA and PWM stimulate different subpopulation of T lymphocytes [11]. It has been reported that ConA is T-cell-specific mitogen and PWM is T-cell-dependent B-cell mitogen in pigs [35]. We determined that the levels of PCV2 Cap mRNA were significantly increased in ConA or PWM stimulated PBMCs compared to non-stimulated cells at 3 and 5 DPI. In addition, the level of PCV2 Cap mRNA in PWM stimulated cells was significantly higher than in ConA stimulated cells at 5 DPI. These findings are in agreement with our previous results [39] that reported an increase in PCV2 replication in PBMCs in the presence of the mitogens, ConA or PWM. These results indicate that stimulation of different populations of lymphocytes results in different levels of viral replication. It has been shown that the severity of PCV2-induced disease is based on the amount of virus present in the pig. Variation in immune stimuli may explain the differences in incidence of PCV2-induced disease between farms and even individual animals.

In order to evaluate whether PCV2 infection affects the ability of cells to proliferate or undergo apoptosis, lymphocyte proliferation and apoptosis indices were assessed. Cell proliferation has traditionally been measured by \(^{3}H\)-thymidine uptaken in cells following stimulation with antigens or mitogens in vitro. However, the ability of CFSE to label lymphocytes intracellularly and track their mitotic activity by progressive two-fold reduction in fluorescence intensity using flow cytometry has proven to be equivalent to the traditional measurement. The advantages of using CFSE labeling is ease of the assay, ability to identify specific subpopulations of proliferating cells and avoiding the use of radioactivity [14].
Results from this study indicated no significant differences in the ability of cells to proliferate when infected with PCV2 at either 3 or 5 days post-stimulation with either mitogen. Thus, PCV2 infection and virus replication did not impact PBMCs proliferation *in vitro*. The results suggest that lymphoid depletion is probably not due to reduced proliferation by PCV2 infection. However, this would need to be further confirmed *in vivo*.

In this study, fluorescein labeled annexin V was combined with 7-AAD to assess the number of viable and/or early apoptotic cells within cultured PBMCs. Annexin V is a Ca$^{2+}$-dependent phospholipid binding protein known to bind to phosphatidylserine on the surface of apoptotic cells [4]. Phosphatidylserine is normally found on the inner, cytosolic side of cell membranes but is exposed on the outside at the early stages of apoptosis [12]. 7AAD is a DNA dye which is excluded by viable cells, but can penetrate cell membranes of dead cells [28]. Results showed that with PWM stimulation, a significantly higher apoptotic index was obtained in virus infected PBMC samples compared to non-infected PBMCs at 3 and 5 DPI, indicating that PCV2 infection and replication increases the level of apoptosis in PWM stimulated PBMCs. This finding provides a possible mechanism for the loss of B cells and a reduction of T cells (CD3+) in lymphoid tissues [31, 33], as well as the reduction of CD4+ T cells and B lymphocytes in peripheral blood of PMWS pigs described in earlier studies [10, 29]. However, no significant differences in the apoptotic index in ConA stimulated PBMCs with or without PCV2 infection was observed in our study. These findings indicate that PCV2 does not increase the apoptosis rate in all stimulated cells suggesting that certain stimulation factors are required for PCV2-induced apoptosis. Previous studies showed that both mitogens stimulate PBMCs to proliferate, but activate different lymphocyte cell populations. The predominant subsets of porcine lymphocytes responding to ConA
stimulation are CD4(+)CD8(+), CD4(-)CD8(+) alpha(hi), CD4(-) CD8(+) alpha(lo) and
gammadelta TCR(+) cells while PWM stimulates CD4(+)CD8(+), CD4CD8(+ )alpha(hi) but
not CD4(-)CD8(+) alpha(lo) or gammadelta TCR(+) cells [11]. In addition, the two mitogens
induce different levels of cytokine production in PBMCs at varying time points after
stimulation [36]. This may be the reason why some studies showed the use of
immunostimulating factors, such as KLH emulsified in incomplete Freund's adjuvant,
vaccines, or non-specific immunomodulating drugs (Baypamun, Bayer, Levewrkusen,
Germany) were able to induce PMWS in PCV2 infected pigs [3, 17, 18, 23] while others did
not when using a commercial vaccine adjuvant as immunostimulating reagent in PCV2
experimentally infected conventional pigs [24]. Further identification of the cells, factors and
mechanisms that trigger PCV2 to induce cell apoptosis is required.

Interestingly, without mitogen stimulation, a significantly lower apoptotic index was
observed in PBMCs samples at both 3 and 5 days post PCV2 infection. It appears that PCV2
infection alone may be able to delay clearance by the host immune system by preventing
cells from undergoing apoptosis. This could explain the presence of PCV2 DNA in tissues
from experimentally infected pigs at 125 DPI [5]. The information obtained in this study
increases our understanding of the mechanism of PCV2 evading from the immune system
and how it persists in infected pigs.

To further study whether the proliferation of PBMCs facilitates PCV2 replication, the
levels of Cap mRNA was assessed in PBMCs that had been stimulated with ConA for 12, 36,
and 72 hours prior to inoculation with PCV2 for 24 hours. No significant differences were
observed in the levels of Cap mRNA in PBMCs at any of the stimulated time intervals prior
to PCV2 infection. This suggests that PCV2 replication in vitro is not dependent on prior
proliferation status of the cells for replication. An alternative explanation would be that productive PCV2 replication requires longer than the 24 hours time period used in this study, though, earlier studies have found differences in replication rate as early as 18 HPI [39]. Future studies should investigate the level of Cap mRNA produced at different time points post-infection and identify the optimum time for the activation factors that will facilitate virus replication.

The level of Cap mRNA in cells induced to proliferate with ConA or PWM stimulation was compared to non-proliferated infected PBMCs. We found that there was no significant difference in Cap mRNA copy numbers in mitogen stimulated proliferating PBMCs compared to non-proliferating cells. Mitogen stimulation also induces macrophage maturation from monocytes in the PBMC population, thus the high level of Cap mRNA in stimulated cells that have not divided may have been from macrophages rather than lymphocytes. It can be hypothesized that macrophages take up infected lymphocytes resulting in further viral replication in the macrophage cell population. In addition, levels of Cap mRNA were significantly greater in PWM stimulated independent of proliferation status compared to ConA stimulated cells. These findings support the earlier findings that PWM stimulated cells are better able to support virus replication compared to cells stimulated with ConA.

In the present study, no significant correlation was found between the copy numbers of Cap mRNA and either the cell stimulation or apoptosis indices. This provides further support for the theory that PCV2 replication is not dependent on cell division. In addition, PCV2 infection alone does not increase the level of apoptosis in infected cells but needs certain stimulation factors to trigger increased levels of viral replication and cell apoptosis.
In conclusion, our data indicates that mitogen stimulation increases virus replication. PCV2 infection and replication do not appear to detrimentally affect the ability of PBMCs to proliferate in response to ConA or PWM stimulation. In addition, no significant differences in cell proliferation occurred in PCV2 infected PBMCs with different stimuli in vitro. Increased apoptosis was associated with PCV2 infection in the presence of PWM stimulation while the apoptosis rate was reduced in cells infected with PCV2 alone. In addition, our results further confirmed our earlier finding that PCV2 replicates in activated lymphocytes. However, stimulation alone is not enough to enhance viral replication. A specific stimulation or trigger is appears to be required, not just cell division or proliferation alone. Determining the role of cellular apoptosis and proliferation plays on PCV2 infection and replication on PBMCs provides important information on the mechanisms involved in the pathogenesis of PCV2 infection and allows us to further determine how this pathogen impacts the immune system of pigs.

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References


Table 1. Quantification of PCV2 spliced capsid mRNA in PCV2 infected peripheral blood mononuclear cells with concanavalin A (ConA) or pokeweed mitogen (PWM) stimulation.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>3 DPI Mean ± Std Dev</th>
<th>5 DPI Mean ± Std Dev</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEMPCV2</td>
<td>4.06 ± 0.89^b</td>
<td>3.22 ± 0.36^c</td>
</tr>
<tr>
<td>ConAPCV2</td>
<td>6.37 ± 0.41^a</td>
<td>5.80 ± 0.25^b</td>
</tr>
<tr>
<td>PWMPCV2</td>
<td>6.33 ± 0.46^a</td>
<td>6.34 ± 0.13^a</td>
</tr>
</tbody>
</table>

MEMPCV2: PCV2 infected PBMCs, ConAPCV2: PCV2 infected PBMCs with ConA stimulation, PWMPCV2: PCV2 infected PBMCs with PWM stimulation

Different superscripts (a, b, c) within each column indicate that values in mean score of treatments for 3 or 5 days post-infection (DPI) is significantly different by least significant difference ($P<0.05$). Values are presented as the mean of log10 mRNA copy number ± standard deviation.
Table 2. The stimulation index of PCV2 infected peripheral blood mononuclear cells with or without concanavalin A (ConA) or pokeweed mitogen (PWM) stimulation.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>3DPI mean ± Std Dev</th>
<th>5DPI mean ± Std Dev</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEM</td>
<td>1.00 ± 0.00&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.00 ± 0.00&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>MEMPCV2</td>
<td>1.38 ± 0.20&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>1.49 ± 0.26&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>ConA</td>
<td>2.67 ± 0.33&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.05 ± 1.10&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>ConAPCV2</td>
<td>2.92 ± 0.31&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.33 ± 3.04&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>PWM</td>
<td>1.79 ± 0.36&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.02 ± 1.87&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>PWMPCV2</td>
<td>1.74 ± 0.15&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.29 ± 2.55&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

MEM: Non-infected PBMCs, MEMPCV2: PCV2 infected PBMCs, ConA: Non-infected PBMCs with ConA stimulation, ConAPCV2: PCV2 infected PBMCs with ConA stimulation, PWM: Non-infected PBMCs with PWM stimulation PWMPCV2: PCV2 infected PBMCs with PWM stimulation.

Different superscripts (a,b,c) within each column indicate that values in mean score of treatments for 3 or 5 days post-infection (DPI) is significantly different by least significant difference ($P<0.05$). Values are presented as the mean of stimulation index ± standard deviation.
Table 3. The viability of PCV2 infected peripheral blood mononuclear cells with concanavalin A (ConA) or pokeweed mitogen (PWM) stimulation.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>3DPI</th>
<th>5DPI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mean ± Std Dev</td>
<td>mean ± Std Dev</td>
</tr>
<tr>
<td>MEM</td>
<td>65.84 ± 8.85&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>49.57 ± 11.14&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>MEMPCV2</td>
<td>69.95 ± 9.34&lt;sup&gt;a&lt;/sup&gt;</td>
<td>68.40 ± 6.34&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>ConA</td>
<td>71.66 ± 3.65&lt;sup&gt;a&lt;/sup&gt;</td>
<td>65.60 ± 12.11&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>ConAPCV2</td>
<td>65.38 ± 6.69&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>62.35 ± 12.39&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>PWM</td>
<td>64.90 ± 3.96&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>46.85 ± 7.87&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>PWMPCV2</td>
<td>53.65 ± 6.24&lt;sup&gt;b&lt;/sup&gt;</td>
<td>31.51 ± 10.96&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

MEM: Non-infected PBMCs, MEMPCV2: PCV2 infected PBMCs, ConA: Non-infected PBMCs with ConA stimulation, ConAPCV2: PCV2 infected PBMCs with ConA stimulation, PWM: Non-infected PBMCs with PWM stimulation PWMPCV2: PCV2 infected PBMCs with PWM stimulation.

Different superscripts (a, b) within each column indicate that values in mean score of treatments for 3 or 5 days post-infection (DPI) is significantly different by least significant difference (P<0.05). Values are presented as the mean of the percentage of live cells ± standard deviation.
Fig. 1. Quantification of PCV2 spliced capsid mRNA in PCV2 infected peripheral blood mononuclear cells (PBMCs) which are proliferated and non-proliferated. PBMCs are stimulated with concanavalin A (ConA) or pokeweed mitogen (PWM) for 5 days and sorted based on the intensity of CFSE staining. The proliferated cells are with low CFSE intensity and non-proliferated cells with high CFSE intensity. Values are expressed as logarithm 10 of PCV2 spliced capsid mRNA copies/10^5 cells. The symbol * represents significant difference between ConA and PWM stimulation at P<0.05.
Fig. 2. Apoptotic index of PCV2 infected peripheral blood mononuclear cells (PBMCs) at 3 (A) and 5 (B) days post-infection. PCV2 infected and non-infected PBMCs were stimulated with concanavalin A (ConA) or pokeweed mitogen (PWM). The symbol * represents significant difference between PCV2 infected PBMCs and non-infected cells at $P<0.05$. 
CHAPTER 6. GENERAL CONCLUSIONS

Porcine circovirus type 2 (PCV2) is considered an important pathogen of swine and is associated with postweaning multisystemic wasting syndrome (PMWS). Lymphocyte depletion of follicular and interfollicular areas together with macrophage infiltration of lymphoid tissues is the hallmark lesions of PMWS-affected pigs (Allan and Ellis, 2000). PCV2 nucleic acid or antigen in PMWS-affected pigs is usually found in the cytoplasm of histiocytes, multinucleate giant cells and other monocyte/macrophage lineage cells. A strong correlation has been observed between the amount of PCV2 nucleic acid or antigen in the tissues and the severity of microscopic lymphoid lesions (Segales and Domingo, 2002). Studies of PMWS-affected pigs found a reduction or complete loss of B cells in lymphoid tissues of PMWS pigs (Rosell et al., 1999). A reduction of T cells (CD3+) and an increase in cells expressing lysozyme (mononuclear-phagocytic cells) was also revealed (Sarli et al., 2001). In peripheral blood, circulating monocyte numbers are increased in PMWS affected piglets, but T cells (CD4+) and B lymphocytes are decreased (Nielsen et al., 2003). Our long-term goal is to determine the mechanism by which PCV2 induces lymphoid depletion and PMWS. Two main objectives were addressed in the research reported here. One was to evaluate PCV2 replication in the immune cell populations in vitro and in vivo, and the other one was to investigate the effects of PCV2 infection and replication on immune cells that had been activated in vitro as well as the relationship between PCV2 replication and cellular proliferation.

Three studies were conducted to evaluate PCV2 replication in the immune cell populations in vitro and in vivo. In the first study, we developed a PCV2-specific reverse transcription polymerase chain reaction (RT-PCR) assay. We demonstrated that our assay
was able to detect the spliced capsid mRNA (Cap mRNA) of PCV2 during virus replication in PK-15 cells and distinguish between PCV1 and PCV2 as no products were amplified from either PCV1 stock or PCV1-infected PK-15 cells. This assay provided a rapid, specific and sensitive technique for detecting type-specific PCV2 while discriminating between replicating PCV2 and PCV2 virions.

In the second study, we further investigated PCV2 replication in peripheral blood mononuclear cells (PBMCs) as well as determining the temporal relationship between cell stimulation with ConA and the level of Cap mRNA using a quantitative real-time RT-PCR assay. This assay was shown to be a sensitive, specific and highly reproducible technique for assessing PCV2 replication. A real-time PCR assay to detect the viral DNA was also performed and the results of the two assays compared. The results showed the Cap mRNA copy number and the PCV2 DNA copy number increased in PBMCs following infection and that stimulation of the PBMCs significantly enhanced both copy numbers in vitro. These findings indicated that PBMCs were capable of PCV2 replication, and stimulation with ConA significantly increased virus replication in vitro. The results support the hypothesis that immune activation may result in increased PCV2 replication (Grasland et al., 2005; Krakowka et al., 2001; Kyriakis et al., 2002; Opriessnig et al., 2003). Moreover, the specific cell populations in which PCV2 actively replicates were identified by labeling cells labeled with FITC-conjugated anti-CD3 antibodies to identify T lymphocytes or FITC-conjugated mouse anti-pig SWC3 antibodies to detect monocytes. An EPICS ALTRA flow cytometer was used to collect each cell population; CD3^+ (T lymphocytes), SWC3^+ (monocytes) and SWC3^- (PBMCs with monocytes removed) cell fractions. It was found that both PCV2 Cap mRNA and viral DNA copy numbers in the cell populations depleted of monocytes (SWC3^-)
and the T lymphocytes population (CD3\(^+\)) were significantly increased compared to the copy numbers in the monocyte population (SWC3\(^+\)). These results suggest that lymphocytes are an important site for PCV2 replication. However, low levels of viral replication products were found in monocytic/macrophage cells which were SWC3\(^+\). Further studies are needed to determine whether these products were due to virus replication or uptake of viral products produced by infected lymphocytes.

In the third study, we investigated PCV2 distribution and replication in tissues and immune cells in early infected pigs and further confirmed the immune cell populations in which PCV2 replicates under \textit{in vivo} conditions. Results of this study found that the viral load and the amount of Cap mRNA levels differed dramatically between tested tissues (bronchial lymph nodes (BLN), superficial inguinal lymph nodes (ILN), lung, tonsil, thymus, spleen, liver and kidney) from the same pig. It was determined that BLN had the highest number of Cap mRNA and also consistently higher levels of viral DNA than any of the other tissues tested at 7, 14 and 21 DPI. These findings indicate that the infection site is important for PCV2 infection and may play a major role in virus spread and persistence in the infected host since PCV2 was inoculated by intranasal route in this study. Cap mRNA was detected in PBMCs only at 13 DPI indicating that PCV2 is able to replicate in PBMCs \textit{in vivo}, though perhaps only transiently when no other pathogens are present. These results also suggest that PBMCs may not be a major source for PCV2 but may be more important in viral dissemination. In contrast, the detection of high levels of PCV2 DNA and replication product in bronchoalveolar lavage (BAL) cells indicates that pulmonary alveolar macrophages (PAM) may be an important site for PCV2 active replication \textit{in vivo}. In contrast to an earlier study, in which it was demonstrated that porcine pulmonary macrophages \textit{in vitro} may not be
a primary target for PCV2 replication (Gilpin et al., 2003; Meerts et al., 2005). The absence of PCV2 replication in their studies may be due to the effect of the *in vitro* culture system on cell activation. More importantly, our results indicated that virus actively replicates in T and B lymphocytes, which appear to be the primary cells that support virus replication. However, the results of detecting PCV2 replication in specific cell populations from BLN mononuclear cells from infected pigs show that both the Cap mRNA and viral DNA levels decreased in T, B lymphocytes at 21 DPI, and monocytes became the cell population with the greatest amount of Cap mRNA and viral DNA. These results suggest that the monocytes may be the site for PCV2 persistence in the infected host. We also found that the local PCV2 specific antibody response consists of IgA and IgM but not IgG. The levels of IgA and IgM appear to be important in restricting PCV2 levels during early infection. Moreover, a strong correlation was present between the levels of Cap mRNA and PCV2 antigen in all tested tissues except the thymus in this study.

Our last study investigated the effect of PCV2 infection and replication on activated porcine PBMCs *in vitro* and the relationship between PCV2 replication and cellular proliferation. The virus replication, cell proliferation, cell viability, and apoptosis in PCV2 infected PBMCs with or without stimuli (concanavalin A (ConA) or pokeweed mitogen (PWM)) were evaluated. This study demonstrated that stimulation with either mitogen increased virus replication in proliferating lymphocytes. However, when we further investigated the relationship between virus replication and cellular proliferation, we found no significant differences between the level of Cap mRNA in PBMCs that had been stimulated 12, 36, and 72 hours prior to PCV2 infection. In addition, there were no significant differences in the amount of Cap mRNA in ConA or PWM stimulated PBMCs that had
proliferated compared to non-proliferated cells. These findings suggested that PCV2 needs a specific stimulation/trigger for facilitating virus replication and is not just dependent on non-specific cell division/proliferation. We also demonstrated that PCV2 infection does not impact the ability of PBMCs to proliferate in response to either ConA or PWM stimulation. Increased apoptosis was found to be associated with PCV2 infection in the presence of PWM not ConA, indicating that PCV2 replication may result in the increase of apoptosis under certain stimulation conditions.

Recent studies have reported that pigs with PMWS have altered cytokine mRNA expression patterns in affected lymphoid tissues (Darwich et al., 2003b) and PBMCs (Sipos et al., 2004). An in vitro study of PCV2 infection of PBMC from healthy and diseased pigs revealed that PCV2 has substantial and specific affects on the function of PBMCs from PMWS pigs in terms of cytokine release (Darwich et al., 2003a). A correlation between PCV2 and MCP-1 and MIP-1 expression in serial sections from lymph nodes of piglets inoculated intranasally with PCV2 has been demonstrated (Kim and Chae, 2003; Kim and Chae, 2004b), suggesting that there is an up regulation of monocyte chemoattractant protein-1 and macrophage inflammatory protein-1 expression by mononuclear cells in response to PCV2. Therefore, future studies should include investigation of cytokine and chemokine profile changes in PCV2 infected immune cell populations in vivo and in vitro. The results of those studies would provide more information on the mechanism by which PCV2 induces lymphocyte depletion and PMWS. Information on the cytokines altered or modulated by PCV2 infection would also increase our understanding of the potential immunosuppression or immunostimulation that has been associated with PCV2 infection in the field.
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